NOVEL ‘HUMANIZED’ MICE TO TEST THERAPEUTICS FOR HUMAN TYPE 1 DIABETES

by

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DEDICATION

I dedicate this work to the memory of two people who had passed this year and have influenced my life and my work; my dear brother Pedro Francisco and a dear friend and colleague Cristina Semino-Mora.

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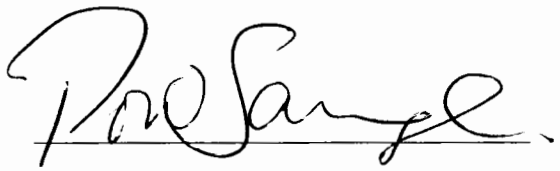

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ABSTRACT

Novel “humanized” mice to test therapeutics for human type 1 diabetes

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The human MHC class II molecules HLA-DR*0401 and HLA-DQ8 have been associated with several cases of T1D as well as other autoimmune disorders. Nevertheless, some studies suggest that there is a potential tolerance effect of the HLA-DR*0401 molecule in humans and transgenically expressed in mice that delays and even exempts them from developing autoimmunity.

Our laboratory has previously reported that genetically-engineered, soluble MHC class II-peptide chimeras prevent, and more importantly reverse early T1D induced by autoreactive T-cells in the NOD mice (16; 60). To validate the therapeutic efficacy of a new soluble HLA-DR4/GAD65 chimera (termed DEF-GAD65 reagent) for human use, we have generated a new humanized mouse model for T1D in the laboratory. Initially, we generated a humanized transgenic NOD (H-2^{g7}) mouse expressing the human MHC class II molecule HLA-DR*0401 (NOD/HLA-DR4 Tg mice), which surprisingly does not develop T1D, nor pancreatic insulitis. We found that NOD/HLA-DR4 humanized transgenic mice have an altered T-cell compartment as compared to their NOD non-

transgenic littermates. We describe a number of experiments demonstrating the resistance of this mouse strain to T1D. Our results strongly suggest that the T1D resistance is due to a combination of several factors such as high number of Foxp3 Treg cells at young age, low INF-γ inflammatory response to polyclonal and antigen-specific stimulation, and an unusually high CD4 to CD8 ratio in the thymus and peripheral lymphoid organs. Our results indicated that the human MHC class II, HLA-DR*0401 has regulatory potency at an early stage of T cell development by altering the thymic output in favor of immune tolerance. In addition to this mouse strain, we generated a NOD/HLA-DR4 strain expressing the human co-stimulatory molecule B7-1(CD80) in pancreatic islets under the rat insulin promoter, by crossing the transgenic NOD/HLA-DR4 and NOD/B7-1 mice. Close to 70% of the F1 hybrids NOD/HLA-DR4/B7-1 double transgenic mice developed an aggressive, spontaneous T1D by 3-4 months after birth, and regardless of gender. In this study we also report that about 75% of the pre-diabetic humanized NOD/HLA-DR4/B7-1 mice treated with a human DEF-GAD65 chimeric reagent remained euglycemic, and that the pancreatic infiltration was stabilized for up to 6 months after treatment interruption by means of Th2/TR-1 polarization in the pancreas.

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LIST OF ABREVIATIONS

Ab(s): Antibody(s)

APC(s): Antigen Presenting Cell(s)

APC: Allophycocyanin. Fluorochrome excited at 600nm it emits light at 660nm

BCR: B Cell Ig Receptor

BrdU: Bromodeoxyuridine. Synthetic nucleoside used as an analogue to thymidine to label cells undergoing cell division.

CD11c: Adhesion glycoprotein expressed in APCs such as dendritic cells and macrophages.

CD19: Type 1 transmembrane glycoprotein expressed by all B cells but not B cells differentiated into plasma cells.

CD25: alpha subunit of the IL-2 receptor

CD28: T-cell receptor co-stimulatory molecule required for naïve T cell activation

CD3: Component of the T cell receptor signaling complex.

CD4: co-receptor for the T-cell receptor that recognizes peptide-MHC class II complexes

CD44: Memory T cell surface marker

CD8: co-receptor for the T-cell receptor that recognizes peptide-MHC class I complexes

cDNA: Complementary DNA

CLSM: Confocal laser scanning microscopy

DAPI: 4, 6' diamidino-2-phenilindole, blue florescent DNA binding molecule.

DEF-: Name given to the peptide-MHC class II chimera developed in Dr. Brumeanu's lab.

DN: Double negative T cells

DP: Double positive T cells.

dTg: double transgenic mouse.

FACS: Fluorescence activated cell sorting. Laboratory technique that uses lasers and microfluidics to identify specific cells based on their fluorescence.

FITC: Fluorescein isothiocyanate fluorochrome

Foxp3: Fork-head box protein 3

GAD65: glutamic acid decarboxylase isoform 65.

GATA3: Trans-acting T cell specific transcription factor.

GFP: Green fluorescent protein

GWAS: Genome Wide Association Studies

H&E: Hematoxylin and Eosin

HA: Hemagglutinin protein from influenza virus

HAI: Hemagglutination Inhibition Assay

HLA: Human Leukocyte Antigen

HRP: Horseradish peroxidase

i.p.: Intraperitoneal

Ig: Immunoglobulin, antibody

IL-: Interleukin

KO: Knock out.

LCK: lymphocyte specific protein tyrosine kinase

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complex

MMTV: Mouse mammary tumor viruses

NOD/RIP-B7: NOD mice carrying a the transgene that codes for costimulatory molecule B7-1 under the Rat Insulin Promoter.

PBMC: Peripheral blood mononuclear cells.

PBS: Phosphate Buffer Saline

pDCs: plasmacytoid Dendritic cells

PE: Phycoerythrin

PerCP: Peridinin clorophyll protein—fluorochrome excited at 490nm and emits at 675nm.

PI3K: Phosphoinositide 3-kinase

pMHC: Peptide-MHC complex.

RAG: Recombination-activating protein, required for T cell receptor and B cell receptor rearrangement.

RAG2 KO: Recombination activating protein 2 knockout mice.

RNA: ribonucleic acid

RPM: Revolutions per minute

RT: Room Temperature

RT-PCR: Reverse transcriptase polymerase chain reaction.

STZ: Streptozotocin.

TcH: T cell hybridoma.

TCR: T cell receptor

TGFβ: Transforming Growth Factor β.

TH: T helper cell

Th1: T helper type 1 cell—known to secrete predominantly IFN-γ and IL-2 generally seen in processes against bacterial, viral, and cancer responses.

Th2: T helper type 2 cell—known to secrete predominantly IL-4, IL-5 and IL-10 generally seen in processes against helminthic infections and allergic reactions.

TNFα: Tumor Necrosis Factor α.

TR1: T regulatory cells expressing Il-10.

Tregs: CD4+Foxp3+T regularoty cells

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Chapter 1: Dissertation Introduction, Hypothesis and Aims

TYPE 1 DIABETES

Type 1 diabetes (T1D) is an autoimmune disorder resulting from the destruction of insulin producing pancreatic β-islets by T-cells (107; 112), ultimately triggering a life threatening state of hypoinsulinemia and extreme hyperglycemia (112). There are several complications that arise after the onset of diabetes such as ketoacidosis, kidney failure, heart disease, stroke and blindness. It has been the goal for many years to find appropriate therapies early in the disease development. Unfortunately, many immune events silently take place early in life with no symptoms before patients seek help from the physician, which is late since the pancreas is severely damaged and thus unable to regulate the blood glucose level (19; 112).

The incidence of T1D is globally rising so that during the past decades reached as much as 5.3% increase in new cases annually in the United States (20). The current trend predicts a doubling in new cases of T1D in European children younger than 5 years between 2005 and 2020, and an alarming prevalence in individuals younger than 15 years will rise by 70% (112). The rising incidence of T1D in the past decade is too rapid to be completely attributed to an increase in genetic susceptibility (113). Reports from different parts of the world indicate that the proportion of youth with T1D having the highest risk human leukocyte antigen (HLA) genotype (DR3/DR4) has not change over time(113). In contrast several changes in lifestyle and environmental factors possibly associated with T1D risk have occurred globally, such as early life infectious disease patterns, changes in diet and physical activity patterns, climate changes, vaccination rates and use of pharmaceuticals (113). The epidemiology of T1D suggests that varying gene–

environment interactions are likely triggering and/or accelerating the autoimmune destruction of the β-cells leading to complete insulin deficiency (113).

The etiology of T1D is still a puzzle mainly because several immunologic events remain silent before the patient shows any signs of disease. Historically many studies point to a genetic component (112) mainly due to early studies that have shown a higher concordance rate of T1D among monozygotic twins than dizygotic twins studied in different populations (46; 53; 77). Though genetic factors were always associated with T1D, they are not sufficient in explaining the increase in previous years of T1D new cases. Certain viruses and nutritional molecules known may trigger β-cell destruction by autoreactive CD8 T cells.

Viral and bacterial infections that may target the pancreatic cells have been also found to trigger T1D in animal models as well as in humans. In animal models, there is evidence for damage to the β-islets caused by viral infections such as encephalomyocarditis virus and coxsackievirus (74; 120). In 2007 Dotta et al.(29) was able to confirm that in humans, coxsackievirus is able to infect the pancreas and cause inflammation triggering T1D. The β-islets could release a variety of antiviral responses for host protection, but in the process may also trigger a host immune-mediated and islet-directed damage (78).

Humoral immunity generating circulating autoantibodies against β-islet antigens such as insulin and glutamic acid decarboxylase (GAD) has been well documented (41; 90; 106), and detection of these autoantibodies serves today as early surrogate markers of disease.

Animal models have been extremely helpful in letting us elucidate the role of cellular mediated immunity in T1D (2; 19; 42; 50; 52; 55). Several experiments performed in non-obese diabetic (NOD) mice, the closest animal model for human T1D, have identified different immune cells involved in pancreatic β-cell destruction, including $CD4^+$ and $CD8^+$ T cells, macrophages, dendritic cells (DC) and natural killer cells (NK) (30; 52; 80; 104; 117).

ROLE OF MHC CLASS II IN T1D

MHC class II molecules are essential for immune activation such as responses against infections and cancer (74). Nonetheless, they are also essential in catalyzing autoimmune disorders by activating self-reactive (autoreactive) T cells. That is because peptide-MHC (pMHC) class II complexes are formed in professional antigen presenting cells (APCs) when they encounter exogenous proteins and engulf them through endocytosis or phagocytosis. Afterwards, the APCs present these protein antigens to the T-cells, which become activated.

It has been well established that pMHC class II complexes activate T-cells through physical contact with the TCR. The activation of $CD4^+$ T-cells by pMHCs requires presentation of 14 to 20 amino acid-long peptide antigens (74; 78). The structure of MHC class II molecules consists of two membrane chains α and β non-covalently linked by strong hydrophobic interactions (74). MHC molecules are indeed essential for the adaptive immune system during education of T cells in the thymus or periphery. Each MHC class II chain contributes to the formation of a groove where the peptide is embedded (78).

The polygenetic factor underlying susceptibility of certain individuals to T1D is associated with a genetic polymorphism occurring in different areas across the genome. In the NOD mouse model there are more than 20 genes called, insulin-dependent diabetes loci (*Idd)* genes that are collectively essential to develop spontaneous diabetes (117). In humans there are also indications of genetic polymorphism found in early studies in two regions of chromosome 6p21 that encodes for MHC class II molecules (HLA-DR, DQ, DP) (*IDDM1* loci) and for insulin in chromosome 11p15 (73; 117).

Currently, there are genome-wide association studies (GWAS) ongoing in different groups across the globe. Early reports from these studies proved that the disease etiology is remarkably complex, since there are more than 40 validated T1D-risk associated loci that explain some 80% of genetic variations in T1D (99). Furthermore, 50% of these alterations were found in the MHC region (*IDDM1*) (99).

Interestingly, a striking similarity shared by mice and humans with T1D was found in the MHC class II I-A β1 chain and HLA-DQ β1 chain respectively, at position 57 where there is a mutation from Asp to Ser residue (57). This report underscores the conservation among species of MHC molecules function and structure. It also stresses on the importance of the NOD mouse as a model to study T1D. Indeed, thanks to all these studies performed in the last half century, it is now generally accepted that an important genetic contributor to the disease are the major histocompatibility (MHC) complexes and the T cells whose functions are activated by the pMHC complexes.

MHC class II molecules are mostly found attached to the external surface of cell membrane of professional antigen presenting cells (APCs) such as dendritic cells and macrophages (78) and B cells. However, some other cells may express MHC class II

molecules in an inflammatory environment (i.e. epithelial cells, microglia, myocytes, fibroblasts, etc.) (12; 14; 64; 98).

Data suggest several different outcomes in T1D onset when the human DR or DQ molecules are co-expressed in different genetic backgrounds, or co-expressed with other MHC class II molecules. Thus, functional studies in a NOD/DQ8 Tg mouse for HLA-DQ or HLA–DR molecules by Taneja et al. (105), and by Gebe et al. (35) in a C57BL/6 (*H-2*^{b})-HLA-DR4 Tg mouse, showed a lack of spontaneous diabetes in these mice even if primed with GAD65 protein (a common β-islet autoantigen in NOD and human T1D), though the mice developed mild insulitis. However, these studies did not address the mechanisms of a possible HLA-DR/DQ interplay that may be responsible for induction of tolerance to the T1D onset. Studies performed by Wen et al. (115) suggests a regulatory mechanism in mice expressing the human HLA-DR4 MHC II molecule. They showed an increased expression of Th2 cytokines that may potentiate a regulatory effect leading to abrogation or delay of T1D in double transgenic DQ8/DR4 mice in a C57BL/6 background. Part of my work presented in this report addresses the potential regulatory role of HLA-DR4 in T1D using our humanized NOD/DR4 transgenic mouse generated in the lab.

ROLE OF T CELLS IN T1D

Even though many argue that CD8+ T cells are essential for triggering β-cell destruction; it remains unclear what the initial culprits of T1D onset are. Several infiltrating cell types have been found to invade the pancreas at different stages of disease development. B cells, dendritic cells, and macrophages serve as the initial activators of autoreactive CD8 and CD4 T cells through peptide presentation by MHC class I and II,

respectively. Also, there are secondary signals that the T-cell receives through the co-stimulatory molecules like CD28 and CTLA-4, and tertiary signals mediated by an array of cytokines such as IL-2, IL-12 and INFγ. These three signals—MHC presentation, co-stimulation and cytokines—are all essential in generating and sustaining an effective immune activation of T helper and CTL cells (37; 112).

A $CD4^+$ T cells major contribution to T1D is perpetuation of an autoreactive CTL activity. After receiving signal from APCs in the pancreatic lymph nodes, activated $CD4^+$ Th1 cells infiltrate the β-islets and command other cells to attack the pancreas by releasing cytokines such as IL-2 and INF-γ. INF-γ production generates a positive feedback loop to increase Th1 cell activity by inducing APCs to release more IL-12, which in turn stimulates the Th1 cell to produce more INF-γ (37).

A certain percentage of activated $CD8^+$ T cells become activated into CTLs and enter islet lesions in greater numbers only when aided by CD4+ T cells and recognize islet antigens. After activation, they enter the pancreatic β-islets becoming the major infiltrating cell population of T cells. After binding to the MHC class I-peptide complex, they form an immunological synapse which prompts them to release cytotoxic enzymes, perforin, granzyme B, and INF-γ (37; 112). These events generate even more destruction within the islets. In turn there is more release of islet antigens that are internalized by surrounding APCs then taken to the pancreatic lymph nodes. APCs will present islet peptides through MHC class I and II to CD8+ and CD4+ T cells respectively. Activated CD4+ T cells will help B cells to become plasma cells releasing an increased amount of autoantibodies, a process known as “epitope spreading” (37). This set of events

eventually overwhelms the pancreatic islets ultimately making them unable to produce insulin enough to sustain normal levels of blood glucose.

SOLUBLE pMHC CHIMERAS ARE PROMISING THERAPEUTICS FOR T1D

Scoring the frequency of self-reactive T cells would be a valuable approach to identify the nature of auto-antigens and to follow the evolution of a T cell mediated autoimmune process. Several decades ago, approaches to score B cells secreting antibodies in blood or *in vitro* were designed based on the ability of the B-cell Ig receptor (BCR) to bind the labeled native antigens (27). Such approaches were inadequate for measuring the frequency of antigen-specific T cells, since the Ig receptor on B cells recognizes linear as well as tridimensional peptide structures (epitopes) on native antigens, whereas the T cell receptor (TCR) on T cells recognizes only short peptide epitopes derived from antigen processing in association with MHC or CD1 gene products. A first attempt to measure the frequency of antigen-specific T cells by flow cytometry used soluble pMHC molecules prepared from APC membranes (92) or recombinant MHC molecules loaded with peptides *in vitro* (36). Later on, Abastado et al. (1) have genetically engineered soluble single-chain MHC class I molecules by fusing the extracellular domain of a murine or human class I molecule to the murine β_2-microglobulin, and then loaded with peptides *in vitro*. These constructs could stimulate IL-2 secretion by peptide-specific T cell hybridomas, and induced activation of cytotoxic CD8 T cells (CTL), but only when they are dimerized or able to form aggregates (1). These studies spearheaded the generation of a set of dimers or multimeric constructs of soluble pMHC-I or pMHC-II by other groups.

The advance in molecular biology opened the door for designing a novel platform for pro-drugs endowed with immunomodulatory properties based on soluble, dimerized pMHC chimeras on immunoglobulin scaffold, in which the peptide is covalently linked via a flexible linker to the β-chain of class II MHC or β_2-microglobulin of MHC class I (28; 89). In our laboratory, dimerization was accomplished by fusion of the αβ chain of the MHC class II molecule to a sequence encoding hinge region, CH2 and CH3 domains of the Fc2γ fragment of murine or human IgG (4). The disulfide bonds between two Fc fragments of the immunoglobulin component allows for the generation of a stable, soluble dimeric molecule as a surrogate of a naturally pMHC molecule expressed on the surface of APCs (4; 32).

In vivo studies on the effects of our genetically engineered, soluble pMHC II dimers (generically termed as Diabetes Eliminating Factor, "DEF") were pioneered in our laboratory in 1995 using a T1D mouse made of double transgenic (dTg) mice obtained by crossing BALB/c mice transgenic for Hemagglutinin (HA) protein of influenza virus expressed in the β-islets under the rat insulin promoter (RIP-HA Tg mice) and BALB/c mice expressing a transgenic HA-specific TCR on T-cells (16). The resulting F1 dTg mice (TCR-HA/RIP-HA dTg mice) develop hyperglycemia in about 10 weeks after birth (16). Treatment of pre-diabetic TCR-HA/RIP-HA dTg mice with a MHC-II-HA110-120 chimera (DEF-HA) prevented diabetes, and more importantly restored normoglycemia in mice with recent onset of diabetes (16). DEF-HA protection relied on the induction of anergy of CD4 T helper cells and stimulation of Th2 and TR-1 secreting IL-10 regulatory cells (16). The CD4 T-cell anergy induced by DEF-HA-mediated TR-1 cells occurred in an antigen specific manner by negative regulation of ZAP-70 and p56lck kinases critical

for early signaling of T cell activation (16). DEF-HA effect on TR-1 cells was supported by several findings including high IL-10 secretion of pancreatic T cells and on the suppressive effect on diabetogenic $CD4^+$ T cells, which could be inhibited by anti-IL-10 antibodies (16). DEF-HA treatment enhanced expression of CD62L, which play an important role in the migration of T cells including TR1 cells into the pancreas where they exert IL-10 mediated suppression of diabetogenic T-cells (16). In the same T1D dTg mouse model our lab demonstrated that DEF-HA treatment protects the syngeneic pancreatic islet transplants against islet-reactive $CD4^+$ T cells and prolonged the survival of transplanted islets (17). Protection of transplanted pancreatic islets occurred by polarization of antigen-specific memory $CD4^+$ T cells toward a Th2 anti-inflammatory response (17).

It was then important to evaluate the therapeutic effect of DEF-like molecules on T cells specific for a dominant β-islet antigen such as the glutamic acid decarboxylase (GAD65), and to determine the bystander tolerogenic effect of DEF molecules on the T cells specific for other minor islet antigens. Unlike the dTg mice in which T1D is mediated by a monoclonal population of autoreactive T cells, polyclonal autoreactive T cells cause T1D in wild type NOD mice as well as in humans. Therefore, NOD mice in which T1D is triggered by a polyclonal population of T-cells were treated with murine constructs of pMHC class II, I-A^{g7}-GAD65 (217-230) (DEF-GAD) or with λ phage peptide (12-24) control (DEF-λ) at an early age (less than 10 days old). Treated mice with a single dose of DEF-GAD65 showed a delay in hyperglycemia onset by roughly 10 weeks when compared with mice treated with control DEF-λ. Another group of pre-diabetic female NOD mice was treated with four doses of 2 μg DEF-GAD65 or control

DEF-λ. Females treated with DEF-GAD65 showed a delay in hyperglycemia while the control DEF-λ treated mice did not (60). It was thus concluded that treatment with a DEF reagent such as DEF-GAD65 targeting a single peptide-specific T-cell population was able to prevent the onset of diabetes in a mouse T1D model in which the disease is triggered by a polyclonal population of T-cells specific for a large number of self-antigens. Furthermore, DEF-induced secretion of IL-10 by TR-1 cells can suppress indiscriminately a number of T-cells specific for different antigens, a mechanism that we called "single-epitope bystander suppression" (see ref.(60)). Furthermore, in T1D reversal type of experiments, a single 5μg administration of DEF-GAD65 in diabetic NOD mice at the early onset (glycemia =250-350 mg/dL) was able to restore normoglycemia within 2 days. The pancreata of normoglycemic mice treated with DEF-GAD65 showed a reduced degree of pancreatic insulitis as compared with those treated with DEF-λ control that remained hyperglycemic (60).

Recently in our lab, human pMHC molecules termed as hDEF have been genetically-engineered for the purpose of testing the therapeutic effect of DEF-like reagents in human T1D. The hDEF reagents were made of DRB1*0401 human MHC class II molecules with covalently linked human GAD65 (271-285) peptide and dimerized through the human IgG1 (hDEF-GAD65) (83). The hDEF-GAD65 molecule was first used to score the frequency of GAD65-specific T cells in the peripheral blood of diabetic patients and to test its capacity to activate *in vitro* GAD65-specific T cells (83). FACS analysis using peripheral T-cells double stained with hDEF-GAD65 and a CD3 antibody showed that GAD65-specific T cells are present in the blood of 19 out 30 patients of 1 to 17 years old with T1D, and in 6 out 7 of their close relatives (83). A

sharp increase in the number of cells binding hDEF-GAD65 reagent was observed upon a short *in vitro* stimulation with GAD65 peptide of peripheral blood cells and reanalyzed by FACS. Using this screening method for diabetic patients it we found that 40-600 T-cells/10^5cells have high avidity for GAD65 antigen (83), a much greater frequency than previously reported by others (10-125/10^5cells) (6-8; 21; 48; 94).

The hDEF-GAD65 also stimulates *in vitro* the secretion of IL-10 by PBMC from patients with T1D, first degree relatives, and some unrelated controls expressing DRB1*0401 molecules (83). In contrast, IL-10 secreted by hDEF-GAD65 stimulated T cells from diabetic patients exhibited a suppressive effect on tetanus toxoid stimulated cells when hDEF-GAD65 and tetanus toxoid were co-incubated with PBMC from diabetic patients (83). These observations indicated again that hDEF-GAD65 reagent can trigger a "single-epitope bystander suppression" of T-cells specific for GAD65-unrelated antigens by means of IL-10 secretion, which strongly suggested that hDEF-GAD65 reagent may regulate a polyclonal population of auto-reactive (diabetogenic) T cells in T1D patients.

HYPOTHESIS

To test the efficacy of our newly engineered hDEF-GAD65 chimeras, we had to generate a new humanized strain of NOD mice in which the human pMHC class II molecules are transgenically expressed, namely the NOD/DR4*0401 Tg mice. To our surprise, the NOD/DR4 Tg mice neither develop T1D, nor can the disease be induced in these mice. Previous studies with mice expressing the human HLA-DR4 and HLA-DQ8 in a C57BL/6 background also revealed a potential protective effect by the HLA-DR*0401

gene by shifting the population of Th1 cells to Th2. *Thus, we hypothesized that unique regulatory events induced by the HLA-DR4 allele take place in our NOD/DR4 Tg mice.*

We have recently expressed in the NOD/DR4*0401 Tg mice resistant to T1D, the co-stimulatory molecule B7-1 under the insulin promoter. More than 70% of NOD/DR4/B7-1 dTg mice develop hyperglycemia in 10-15 weeks after birth, regardless gender. *Based on observations and data presented above, we further hypothesize that treatment of NOD/DR4/B7 dTg mice with hDEF reagents will delay T1D in these mice by inducing IL-10-secreitng TR-1 cells in the pancreas*.

AIMS WITH RATIONALE

1. **We have addressed the mechanism of HLA-DR4-induced tolerance to T1D in humanized NOD/DR4 Tg mice.** We found significant immunologic differences between the NOD/DR4 Tg mice resistant to T1D and their NOD non transgenic littermates that develop T1D. Specifically, we found a higher frequency of Foxp3+ Treg cells at an early age and a significantly lower frequency of $CD8^+$ T cells in the NOD/DR4 Tg mice resistant to T1D. Based on our preliminary data, we demonstrated that the human *HLA-DR*0401* allele exerts a regulatory (protective) effect against T1D development. In chapter 2, I am presenting a detailed series of experiments used to investigate the role of several T-cell subsets in T1D progression such as the $CD4^+$ Th cells, Foxp3+ Treg cells, TR-1 cells, and the recently described DN (CD3+4-8-double negative) T regulatory cells in our lab (31).
2. **We have assessed the therapeutic efficacy of a soluble pMHCII-GAD65 chimera in T1D using our newly generated NOD/DR4/B7-1 double**

transgenic mice that develops spontaneous T1D. We and others reported that soluble pMHC II chimeras engineered on a murine IgG scaffold can prevent and reverse early T1D in animal models. Herein, we carried out pilot experiments to address whether treatment with a human pMHC II (HLA-DR4) chimera can protect against T1D. This work is described in detail in chapter 3. The work presented below has been compiled in two manuscripts submitted for publication, as follows:

Chapter 2: Multi-point T-cell regulation by human MHC class II (*HLA-DR*0401)* allele obviates the *I-A^{g7}* diabetogenicity

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ABSTRACT

Background: Human MHC class II (HLA) molecules are involved in the development of several autoimmune diseases including type 1 diabetes (T1D). The HLA-humanized mice offer the advantage of studying the function of individual HLA alleles expressed in an autoimmune background.

Methodology/Principal findings: The *HLA-DR*0401* allele transgenically expressed in a NOD (*H-2^{g7}I-E^{null}*) diabetogenic background (NOD/DR4 transgenic mice) abrogated the disease in the context of multiple T-cell alterations such as: (i) skewed CD4/CD8 T-cell ratio, (ii) decreased size of CD4$^+$CD44^{high} T memory pool, (iii) aberrant TCR Vβ repertoire, (iii) increased neonatal number of Foxp3$^+$ and TR-1$^+$ regulatory cells, and (v)

reduced IFN-γ inflammatory response *vs.* enhanced IL-10 suppressogenic response to polyclonal T-cell stimulation. The NOD/DR4 T-cells were unable to induce or suppress diabetes in a NOD/RAG deficient system. In contrast, antigen-specific B-cell responses remained unaltered in the NOD/DR4 Tg mice.

Conclusions/Significance: This study argues for regulatory functions of *HLA-DR*0401* allele that can unable the T-cell compartment to uphold the T1D development.

Keywords: NOD humanized mice, HLA-DR*0401, T-cell regulation, Type 1 Diabetes suppression.

INTRODUCTION

Type 1 diabetes (T1D) is an organ-specific autoimmune disease mediated by a polyclonal self-reactive population of T-cells leading to the destruction of pancreatic insulin-secreting ß-cells. The disease is strongly associated with gene polymorphism at twenty insulin-dependent diabetes (*Idd*) loci, among which the *Idd1* locus encoding for MHC class II molecules (HLA in humans) plays a critical role (74; 75; 110). The polymorphism in MHC genes accounts for 40-50% of the T1D familial aggregation (54; 76) with the highest risk linked to complementation between the DQA1*0501 and DQB1*0302 genes (54). The most common trans-complementing haplotype found in T1D patients is the DRA1*0101-DRB1*0401 (DR4) and DQA1*0301-DQB1*0302 (DQ). We found that 30% of T1D Caucasian patients express the HLA-DR*0401 allele (83). Studies performed on a large number of families with different ethnicities suggested that the proportion between DR4 and DQ alleles (odds ratio, OR) is an important parameter to predict the HLA-dependent susceptibility *vs*. protection in T1D. Thus, a DR4:DQ8 OR higher than 1 would indicate low risk, whereas a lower OR than 1

would indicate protection (97). It thus appears that the DQ8 predisposition to T1D is regulated by its closely linked DR alleles (70; 112) such as DRB1*0401, 0403, and 0405 (34).

HLA-humanized mouse models for autoimmunity offer the advantage of studying the function of individual HLA alleles and generation of murine T-cell hybridomas restricted to human epitopes. The concern of CD4 species barrier in the HLA-humanized mice (9; 95; 114) for a proper interaction of the murine CD4 with HLA molecules has been recently underrated by studies demonstrating the presence of murine CD4 T-cell responses to antigen challenge in a HLA-specific fashion (118; 119). Conversely, transgenic human CD4 molecules were able to rescue the development and function of murine CD4 T-cells in mice lacking endogenous CD4 molecules (47). Generation of murine/human MHC class II chimeric molecules preserving the murine CD4 binding domain and the HLA antigen binding site (44; 69) has minimized the concern of CD4 species barrier, and increased the value of HLA-transgenic mice for functional studies.

Herein, we used a new transgenic (Tg) NOD mouse expressing the MHC class II HLA-DRA1-DRB1*0401 molecule to study its role in autoimmune diabetes. Data presented argues for T-cell regulatory functions of HLA-DRA1-DRB1*0401 molecules that are able to obviate the disease development in the NOD diabetogenic background. The mechanisms underlying the HLA-DR4-induced resistance to T1D are discussed.

METHODS

Ethics statement

Mice were housed in pathogen-free conditions at the USUHS/LAM facility. Experiments and care/welfare were in agreement with local and federal regulations and an approved protocol by the USUHS IACUC committee.

Mice

The humanized NOD/DR4 Tg strain was generated by inter-strain breeding. The C57BL/6 mice deficient for MHC class II molecules (H-2^{b+}, IAβ^{-}/IEα^{-}*)* and transgenic for a human/murine chimeric HLA-DR4-IE molecule (HLA-DRA-IE^{d}α/HLA-DRB1*0401-IE^{d}β) (Taconic, NY) were backcrossed for 12 generations into the NOD diabetogenic background (IA^{g7+}, IE^{d}_{null}, H-2^{d+}) to generate the NOD/DR4 Tg strain (HLA-DRA-IE^{d}α/HLA-DRB1*0401-$IE^{d}$$\beta^{+}$, IA^{g7+}, IE^{d}_{null}, H-2^{d+}). Full recovery of the NOD background was confirmed by microsatellite analysis, and selection of NOD/DR4 Tg offspring was carried out by PCR using specific primers for HLA-DR*0401 (forward: GTTTCTTGGAGCAGGTTAAACA; reverse: CTGCACTGTGAAGCTCTCAC). Internal control PCR primers for DNA quantification were specific for mouse IgG3 gene (forward: ACAACAGCCCCATCTGTCTAT; reverse: GTGGGCTACGTTGCAGATGA C). The NOD/Rag1 KO mice used in adoptive transfer experiments were purchased from Jackson Laboratories (Bar Harbor, ME).

In Vivo Protocols

Adoptive cell transfer experiments.

Various numbers of splenic T-cells (10-50x10^{6} cells) from NOD/DR4 Tg mice and/or 5-6 month-old, hyperglycemic non transgenic NOD littermates were infused i.p. either alone or together into 3 month-old NOD Rag1 KO female recipients. Control diabetes groups were the NOD Rag1 KO recipient mice infused i.p. with 10x10^{6} or

$50x10^6$ splenic cells from 5-6 month-old, hyperglycemic NOD mice. In some experiments, $50x10^6$ splenic cells from diabetic NOD wt mice were infused i.p. into NOD/DR4 Tg mice. The onset of diabetes in recipient mice was monitored bi-weekly based on blood glucose levels using Accu-Check glucose meter and glucose test strips (Roche, Indianapolis, IN, USA). Mice were considered diabetic after two consecutive readings higher than 200 mg/dL.

Cyclophosphamide treatment.

Three month-old, normoglycemic NOD non Tg females and their NOD/DR4 Tg female littermates were injected i.p. with 3 doses of cyclophosphamide (200 mg/kg) in saline (Baxter, Deerfield, IL) every other fifth day, and followed bi-weekly for the hyperglycemia onset.

Viral immunization.

Three month-old, normoglycemic NOD non Tg females and their NOD/DR4 Tg female littermates were immunized i.p. with 50 μg of UV-inactivated A/PR/8/34 influenza virus in saline (Charles River, Wilmington, MA) or saline alone. Three weeks later, blood serum was collected from individual mice and measured by ELISA for PR8-specific antibodies and by Hemagglutination Inhibition Assay (HIA) for titers of anti-viral neutralizing antibodies as we previously described (100). Experiments were carried out at USUHS under the MED-11-655 and MED-11-805 IACUC protocols and according to EU Directive 2010/63/EU.

In vivo BrdU labeling.

2 month-old NOD/DR4 Tg mice and NOD non Tg littermates were injected i.p. with 0.8 mg of BrdU, and spleens were harvested 1 hr post injection. Spleen cells were

prepared for flow cytometry analysis following the manufacturer's instructions BrdU-FITC flow kit staining protocol (Cat No 559619 BD Pharmingen, San Diego, CA), and stained with an CD4 Ab-AlexaFluor or CD8 Ab-PE conjugate for 30min BD PharMingen). Cells were then fixed, permiabilized, treated with DNAse, incubated with BrdU Ab-FITC conjugate, and analyzed in a BD LSR-II flow cytometer as described below.

Glucose tolerance test

Mice were fastened overnight, injected i.p. with 60 mg of glucose in saline. Blood glucose levels were monitored at various time-points after injection using an Accu-Check glucose meter and glucose test strips (Roche).

Single-Cell Flow cytometry

Single-cell suspension of splenocytes (10^6 cells) harvested from individual mice from different groups were stained 30 min at 4°C for various cell surface markers using specific Ab-dye conjugates or their isotype controls. In some experiments, splenocytes were stained for TCR Vβ families and co-stained for intracellular IL-10, or for intranuclear *Foxp3* protein using specific antibody-dye conjugates and their isotype controls (BD Biosciences, San Jose, CA). Some 10^4-10^5 cell events were acquired in FACS experiments using a LSR II Becton-Dickinson instrument equipped with the WINLIST analysis software (Verity, Topsham, ME, USA), or with a BD FACS DIVA software (BD Biosciences).

Antibody Assays

Anti-PR8 influenza antibody response in pre-diabetic NOD non Tg mice and NOD/DR4 Tg littermates were measured by ELISA and Hemagglutination Inhibition

Assay (HIA), as we previously described (100). Duplicate 1/100 serum dilutions in 0.1% BSA/PBS from individual mice in each group were measured for PR8-specific Ab titers in ELISA using anti-mouse IgG (H+L) Ab-Biotin conjugate (Abcam, Cambridge, MA) in PR8 virus-coated 96-well plates. Bound IgG Ab-Biotin conjugates were revealed by a streptavidin-HRP conjugate (Jackson ImmunoResearch) developed in 3,3´,5,5´-tetramethylbenzidine (TMB) substrate (BD Biosciences). The OD units corresponding to the Ab titers were measured at 450 nm in a 96- well ELISA reader (Molecular Devices Vmax, Sunnyvale, CA). The Hemagglutination Inhibition Assay (HIA) was carried out in the same individual mice from each group using serial dilutions of serum pre-treated overnight at 37ºC in phosphate buffer (containing 5mM of CaCl2, pH=7.4) with neuraminidases (from Arthrobacter ureafaciens and Clostridium perfringens, 50 mU each/ml, Calbiochem, Bibbstown, NJ, USA). Neuraminidase-treated serum dilutions (50 μl/sample) were then incubated in 96-well plates for 4 h at room temperature with 50 μL of 1% sheep red blood cells in saline (Innovative Research, Novi, MI) in the presence or absence of 25 μL of UV-inactivated PR8 virus (3 μg/well). The HIA titers were expressed as the one serum dilution above the first dilution showing inhibition of hemagglutination.

Cell isolation

CD4 T-cells were isolated from the spleen of mice by negative sorting on CD4 mouse column kits (BD PharMingen). In some experiments, splenic $CD3^+$ T-cells were sorted on CD3 magnetic immunobeads according to the manufacturer's instructions (Miltenyi Biotech), washed, and rested at 37ºC and 5% CO2 for 24 h in RPMI medium containing 10% FCS before use. Dead cells were removed by 10 min centrifugation at

1,000 rpm and cell viability in trypan blue staining was measured microscopically. Cell preparations with viability higher than 85% were used in the assays.

Cytokine measurement

Single-cell suspensions of splenocytes from individual mice in each group were incubated at 5% CO_2 and 37°C in RPMI complete media containing 10% FCS and CD3/CD28 mAbs (2 μg/mL each/10^6 cells/well), or recombinant GAD65 protein (10 μg/10^6 cells), or synthetic $GAD65_{555\text{-}567}$ peptide (20 μg/10^6 cells), or in medium alone. Cells were cultured in flat-bottom 96-well plates for 24 h to measure IL-2 secretion, and respectively 72 h to measure the IL-4, IL-10, and IFN-γ secretion. Cytokine secretion in the cell culture supernatants was measured in triplicate wells using Multiplex mouse cytokine kits and a Luminex instrument (Luminex Corporation, Austin, TX). A 5-parameter logistics model equation was used to measured cytokine concentration according to the manufacturer's instructions (MasterplexQT software, Miraibio, San Francisco, CA).

Confocal laser scanning microscopy (CLSM)

Plastic adherent cells (APCs) from the spleen of non-transgenic NOD mice and NOD/DR4 Tg littermates were co-stained for 30 min at 4°C on ice with HLA-DR4-FITC and I-A^d-Alexa Fluor conjugates (1.5 μg each/10^6 cells) (BD Biosciences). Cells were washed twice in PBS/BSA 1% and mounted onto glass slides using Vectashield containing DAPI (Vector Laboratories Inc., Birmingham, CA). Co-localization of HLA-DR4 and I-A^{g7} molecules and their density at the single-cell level was measured by means of fluorescence intensity for each Ab-dye conjugate captured as a 2D image with a

ZEISS 710 Confocal Laser Scanning Microscope equipped with ZEISS ZEN 2009 analysis software (Thornwood, NY, USA).

Histology and Immunohistochemistry

The pancreas from individual mice in each group was fixed overnight in 10% phosphate-buffered formalin and embedded in paraffin. Serial 5μ sections of pancreata were stained with Hematoxylin-Eosin (H&E) to identify infiltrating lymphocytes, or immunostained with a rabbit anti-insulin antibody (Santa Cruz Biotech, Santa Cruz, CA) revealed by a goat anti-rabbit IgG-HRP conjugate (Southern Biotechnologies, Birmingham, AL) to estimate the extend of insulin secretion and intra-islet distribution of insulin granules. Between 15 and 20 pancreatic β-islets for each pancreas were analyzed microscopically.

Biostatistics

T1D incidence in NOD non Tg mice and NOD/DR4 Tg littermates within the same group and between groups of mice under various experimental conditions was determined by the nonparametric Kaplan-Meier test for which p^* values less than 0.05 were considered significant. Individual variations in the anti-PR8 virus-specific Ab and HIA titers in the influenza immunized mice and the intra-assay variations in the cytokine assays were measured by Student's *t*-test and expressed as mean ± standard deviation (SD) at 99% interval of confidence.

RESULTS

Human HLA-DR*0401 and murine I-A^{g7} molecules are co-expressed on APCs in NOD/DR4 Tg mice

The HLA-DR*0401 molecules were expressed on about 30% of splenic $CD11c^{+}CD19^{-}$dendritic cells and 40% of splenic $CD19^{+}$ B-cells in the NOD/DR4 Tg mice regardless the gender, whilst $I\text{-}A^{g7}$ expression was detected on ≈ 90% of splenic $CD11c^{+}$ $CD19^{-}$ dendritic cells and 98% of $CD19^{+}$ B-cells in both NOD/DR4 Tg mice and NOD non Tg littermates (Fig. 2.1A). FACS analysis suggested that a large number of APCs co-express both the HLA-DR4 and $I\text{-}A^{g7}$ molecules in the NOD/DR4 Tg mice. Indeed, CLSM analysis at single-cell level in enriched APCs preparations from the spleen of NOD/DR4 Tg mice showed that most of human and murine MHC class II molecules are co-expressed (Fig. 2.1B) and that the HLA-DR4 molecules have various levels of cell density (fluorescence intensity) (Fig. 2.1B**,** upper panels). Few dendritic cells (≈ 1.5%) and B cells (≈ 0.15%) showed a sole expression of human HLA-DR4 molecules (Figs. 2.1A and 2.1B, lower panels). These results indicated that the HLA-DR*0401 expression level on the NOD/DR4 APCs was relatively high and it did not interfere with the expression level of $I\text{-}A^{g7}$.

The NOD/DR4 Tg mice are resistant to spontaneous and inducible diabetes

Pancreatic insulitis is revealed as a lymphocyte infiltration in the pancreatic β-islets and it starts developing some 3-5 weeks after birth, and the hyperglycemia onset can be detected 4-6 months after birth in the NOD wild (wt) females. In contrast to the NOD non Tg littermates, none of NOD/DR4 Tg females and males (n=167) developed pancreatic lymphocyte infiltration (Fig. 2.2A) or hyperglycemia. Consistent with the results of microsatellite analysis, the spontaneous occurrence of diabetes in NOD non Tg littermates clearly demonstrated that the diabetogenic NOD background was fully recovered during the cross-breeding protocol. The insulin secretory function of β–cells in

NOD/DR4 Tg mice remained unaltered according to the glucose tolerance test, whereas the pre-diabetic and diabetic NOD non Tg littermates showed a delay in restoring euglycemia after the glucose load (Fig. 2.2B).

To find out whether diabetes can be induced in the NOD/DR4 Tg mice, we treated the mice with cyclophosphamide, a cytostatic drug known to accelerate T1D onset in NOD wt mice (reviewed in ref (70)). None of the cyclophosphamide-treated NOD/DR4 Tg mice developed pancreatic insulitis or hyperglycemia during a 4-month follow-up. In contrast, 85% of non Tg littermates showed early hyperglycemia, some 5 weeks after the last cyclophosphamide injection (Fig. 2.2C). These results indicated that the NOD/DR4 Tg mice, but not their NOD non Tg littermates are resistant to spontaneous and inducible T1D.

T-cells from NOD/DR4 Tg mice are neither diabetogenic nor tolerogenic

To find out whether the T-cells from NOD/DR4 Tg mice may exhibit diabetogenic effects or interfere with the diabetogenic function of T-cells from NOD wt mice, groups of NOD/Rag1 KO mice were infused i.p. with splenic T-cells from NOD/DR4 Tg mice ($50x10^6$ cells/mouse), or co-infused i.p. with a 1:1 or 5:1 mixture of splenic T-cells from NOD/DR4 Tg mice and hyperglycemic NOD wt mice. Control groups were the NOD/Rag1 KO mice infused with splenic T-cells from hyperglycemic NOD wt mice ($10x10^6$ or $50x10^6$ cells/mouse) (Fig. 2.2D). None of NOD/Rag1 KO recipients of T-cells from NOD/DR4 Tg mice developed hyperglycemia or pancreatic insulitis. In contrast, NOD/Rag1 KO mice infused with T-cells from hyperglycemic NOD wt mice, or with mixtures of T-cells from NOD/DR4 Tg mice and hyperglycemic NOD wt mice, developed hyperglycemia within 4 to 6 weeks. These experiments demonstrated

that the T-cells from NOD/DR4 Tg mice do not exert diabetogenic or tolerogenic effects *in vivo*.

NOD/DR4 Tg mice and their non Tg littermates express a different T-cell repertoire

It has been shown that the nature of MHC class II-peptide complexes can shape the phenotype and function of T-cells (43; 91). A number of T-cell aberrations have been revealed in the NOD/DR4 Tg mice in this study. First, the frequency of $CD4^+$ thymocytes was higher (*p = 0.006), whereas the frequency of $CD8^+$ thymocytes was lower (*p = 0.009) in the NOD/DR4 Tg mice than in non Tg littermates (Fig. 2.3A, upper panels), though the absolute numbers of thymocytes was comparable in both strains (Fig. 2.3B, upper left panel). Furthermore, the frequency of CD4 mature T-cells in the spleen of NOD/DR4 Tg mice was also higher (*p = 0.0003), whereas the frequency of CD8 mature T-cells was lower than in non Tg littermates (*p = 0.0003) (Fig. 2.3A, lower panels) in the context of comparable absolute numbers of splenic T-cells between the two groups (Fig. 2.3B, lower right panel). According to these measurements, a significant CD4/CD8 skewed ratio in the thymus and spleen of NOD DR4 Tg mice was constantly detected in the NOD/DR4 Tg mice (Fig. 2.3B, upper and lower right panels). This raised the question of whether unusual apoptosis or homeostatic rate of T-cells may play a role in the CD4/CD8 skewed ratio in NOD/DR4 Tg mice.

Analysis of early and late apoptosis of CD4 and CD8 mature T-cells showed no difference in the extend of cell death between the NOD/DR4 Tg mice and their non Tg littermates (Fig. 2.3C). However, the fate of splenic CD4 and CD8 T-cells determined in BrdU experiments was different between the two groups of mice, as the homeostatic rate of mature CD4 T-cells was ≈ 40% faster, and the homeostatic rate of mature CD8 T-cells

was ≈ 80% slower than in age-matched, non Tg littermates (Fig. 2.3D). Thus, the thymic output and rate of homeostasis were much responsible for a CD4/CD8 skewed ratio in the NOD/DR4 Tg mice.

We next questioned whether a skewed CD4/CD8 thymic output may also occur in different genetic backgrounds expressing the HLA-DR*0401 transgene, and whether the presence of endogenous expression of murine MHC class II molecules may interfere with the CD4/CD8 regulatory effect of HLA-DR*0401 transgene. HLA-DR*0401 expression in the C57BL/6 (*H-2*^{b}) background skewed the CD4/CD8 thymic output as observed in the NOD background, regardless the endogenous co-expression of murine MHC class II molecules. This was also the case in the spleen of NOD and C57BL/6 mice, regardless the co-expression of endogenous murine MHC class II molecules (Fig. 2.4). These results indicated that the human HLA-DR*0401 allele exerts regulatory effects on the CD4/CD8 thymic output and peripheral CD4/CD8 ratio in different genetic backgrounds independently of the endogenous expression of the murine MHC class II molecules.

Since the CD4 and CD8 T-cell recall response to self-antigens are important in T1D pathogenesis, we next compared the size of CD44^{high} CD4^{+} and CD8^{+} T memory pool in NOD/DR4 Tg mice (resistant to T1D) and NOD non Tg littermates (prone to spontaneous T1D). The frequency of both the CD44^{high} CD4^{+} and CD44^{high} CD8^{+} T memory cells was elevated in the NOD non Tg mice as compared with their DR4 Tg littermates (28.9 ± 0.87% *vs*. 22 ± 1.69%, and respectively 42.3 ± 10.9% *vs*. 28.86 ± 3.79%) (Fig. 2.5), suggesting a less active process of T-cell stimulation by self-reactive proteins in the NOD/DR4 Tg mice.

The NOD/DR4 Tg mice express an aberrant TCR Vβ repertoire

Like in the parental C57/BL6 (Abb KO)/HLA-DRA1-IEα_2/DRB1*0401-IEβ_2 Tg mouse (44) used to generate our NOD/DR4 Tg strain, the TCR Vβ5 family was poorly expressed and the Vβ17 family was deleted in the NOD/DR4 Tg mice. In contrast, the Vβ11 and Vβ12 families were highly expressed in the NOD/DR4 Tg mouse whilst deleted in C57/BL6 Abb KO/HLA-DRA1-IEα_2/DRB1*0401-IEβ_2 Tg mouse. The Vβ5 family was poorly expressed (*p=0.01), whereas the Vβ6, Vβ12, and Vβ14 families were higher expressed in the NOD/DR4 Tg mice than in non Tg littermates (*p=0.002, 0.02, and 0.003 respectively) (Fig. 2.6). Thus, as in the case of other human HLA transgenes expressed in mice, the HLA-DR*0401 transgenic expression in NOD mice induced quantitative alterations in some TCR Vβ families.

IFN-γ and IL-10 responses are altered in NOD/DR4 Tg mice

IFN-γ pro-inflammatory T-cell responses to β-cell antigens can damage the pancreatic islets, while the IL-10 responses can suppress the inflammatory process (60; 81). Splenic T-cells from the NOD/DR4 Tg mice showed a significantly reduced IFN-γ response and an increased IL-10 response to polyclonal stimulation with CD3/CD28 Abs (*p=0.028) (Fig. 2.7, left panel). Similarly, the IFN-γ response to recombinant GAD65 protein (major autoantigen in T1D (40; 116) was significantly low in the NOD/DR4 Tg mice as compared with their non Tg littermates (*p=0.0065) (Fig. 2.7, middle panel). Furthermore, stimulation of NOD/DR4 spleen cells with a major diabetogenic peptide (GAD65/67$_{555\text{-}567}$ (35)) failed to induce IL-2 secretion (Fig. 2.7, right panel). There was no significant alteration in the IL-4 response to the CD3/CD28 polyclonal stimulation or rGAD65 protein between the two groups of mice.

These results revealed first, an intrinsic deficiency in the IFN-γ inflammatory response and a high IL-10 suppressogenic response to polyclonal T-cell stimulation in the NOD/DR4 Tg mice. Secondly, the T-cell response to a major GAD65/67$_{555\text{-}567}$ diabetogenic epitope was absent in the NOD/DR4 Tg mouse.

The NOD/DR4 Tg newborns have a stronger Treg compartment than the NOD non Tg littermates

The anti-diabetogenic roles of Foxp3 T regulatory cells (Treg) and IL-10-secreting TR-1 cells in T1D have been widely demonstrated (16; 45; 60; 63; 85). We found a significantly high number of Foxp3$^+$ T-cells in the NOD/DR4 Tg newborns as compared with their aged-matched, non Tg littermates (*p=0.035). However, the size of Foxp3$^+$ T-cell pool in the adulthood was comparable to that of non Tg littermates (Fig. 2.8A). In both groups of mice, Foxp3 expression was associated with all TCR Vβ families except Vβ17a. The highest Foxp3 expression in the NOD/DR4 Tg mice was by Vβ11 (*p=0.014) and the lowest by Vβ14 family (*p=0.047) (Fig. 2.8B). Interesting enough, these data revealed first, that the expression of *HLA-DR*0401* allele in the NOD diabetogenic background favored the development of a high number of Foxp3$^+$ Treg cells in the neonatal stage, which coincides with a sensitive time-window when the lymphocyte infiltration occurs in pancreas. Secondly, *in vitro* polyclonal stimulation of spleen cells with CD3/CD28 Abs in the presence of exogenous IL-2 increased significantly the number of Foxp3$^+$ CD8 mature T-cells preferentially in the NOD/DR4 Tg mice (*p=3.8x10^{-6}) (Fig. 2.8C).

In contrast to the differential expansion of Foxp3$^+$ Treg cells in NOD/DR4 Tg neonates, the T-cells from both groups of mice showed comparable IL-10 synthesis in the

neonatal and adult stages (Fig. 2.9A), with T-cells expressing TCR Vβ3 and Vβ13 having the highest IL-10 synthesis (Fig. 2.9B). The fact that the IL-10+ T-cell frequency was comparable in both groups of mice but the polyclonal IL-10 response was higher in the NOD/DR4 Tg mice, indicated that the T-cells from NOD/DR4 Tg mice have not only a poor IFN-γ inflammatory response (Fig. 2.7A), but also a suppressogenic response.

We have recently reported that the splenic $CD3^{+}4^{-}8^{-}$ double negative (DNCD3) T-cells from NOD wt mice represent a unique T-cell population that can delay the onset of diabetes in NOD/Scid mice infused with diabetogenic T-cells by a mechanism of differentiation into IL-10-secreting TR-1 cells in the pancreas (31). Analysis of DNCD3 T-cells showed no quantitative difference between the NOD/DR4 Tg mice and their non Tg littermates (Fig. 2.S1), which ruled out the role of these regulatory cells in T1D resistance of NOD/DR4 Tg mice.

The NOD/DR4 Tg mice have unaltered antigen-specific B-cell responses

Since the NOD/DR4 Tg mice showed a number of quantitative and qualitative T-cell aberrations critical for the development of diabetes, we lastly questioned whether the B-cell responses are also altered in these mice. Both groups of mice were immunized with influenza viral antigens (UV-inactivated A/PR8/34 influenza virus), and 21 days later the virus-specific IgG antibodies and their ability to neutralize the virus were measured by ELISA, and respectively Hemagglutination Inhibition Assay (HIA). The amount of total IgG before immunization and titers of virus neutralizing antibodies in sera were comparable in both groups of mice (Fig. 2.S2). Thus, in contrast to the phenotypic and functional alterations in the T-cell compartment, the antigen-specific B-cell responses in the NOD/DR4 Tg mice remained unaltered.

DISCUSSION

This study revealed a number of alterations in the T-cell compartment of NOD mice upon transgenic expression of human MHC class II HLA-DR*0401 allele. These alterations in the NOD/DR4 Tg mouse occurred in context of T1D resistance.

The NOD/DR4 Tg mice showed a skewed CD4/CD8 T-cell ratio at the expense of CD8 T-cells due to deficient CD8 thymic output and CD8 T-cell homeostasis. Skewed CD4/CD8 T-cell ratios have been reported in some mouse strains (3; 50), rats (26) and humans (93). A suggested mechanism refers to the MHC polymorphism in discrete regions encoded by exon 3 of human DRα or murine I-Eα chains (33; 55). However, this mechanism has been ruled out, since the HLA-DRA1-DRB1*0401/I-$E^{d}_{\alpha 2\beta 2}$ chimeric transgene in the NOD/DR4 Tg mice does not incorporate the human and murine exon 3 of the HLA-DR gene. It has been also suggested that the TCR specificity (38; 58) and nature of HLA haplotype (3; 26; 50) can dramatically change the CD4/CD8 T-cell ratio. Another possible mechanism of a skewed CD4/CD8 ratio may refer to newly-generated TCR specificities by HLA-DR4/murine I-A^{g7} α/β hybrids though this is unlikely to occur in NOD/DR4 Tg mice, since no such miss pairing events were detected in the parental C57BL/6 (Abb KO), HLA-DRA1-DRB1*0401/I-$E^{d}_{\alpha 2\beta 2}$ Tg strain (44) used to generate our NOD/DR4 Tg strain. Since the TCR specificities are selected in thymus through MHC-peptide presentation (23; 43; 91; 96), and the human HLA molecules expressed in mice can generate new TCR specificities by presenting a different set of self-antigens than the murine endogenous MHC class II molecules (52), it appears that this is the most likely mechanism leading to a skewed CD4/CD8 T-cell ratio in the NOD/DR4 Tg mice.

Since the HLA-DR*0401 and murine I-A^{g7} molecules are co-expressed on the NOD/DR4 APCs without altering the number of APCs expressing I-A^{g7} or density of I-A^{g7} molecules on cell surface, further investigation would be required to determine whether competition for peptides binding between the two MHC class II molecules may occur at the single-cell level, and whether this may lead to "diluted" I-A^{g7} presentation of diabetogenic peptides. Our data showed that the NOD/DR4 T-cells failed to induce diabetes in NOD/RAG deficient mice, and did not respond to a major $GAD65_{555-567}$ diabetogenic epitope. Aberrations in TCR specificities for $GAD65_{555-567}$ reactivity due to CD4 species barrier have been ruled out, because the murine CD4 binding site is preserved in the HLA-DRA-$IE^d\alpha_2$/HLA-DRB1*0401-$IE^d\beta_2$ chimeric molecule in the NOD/DR4 Tg mouse. The NOD/DR4 T-cells responded to CD3/CD28 polyclonal stimulation by lower IFN-γ secretion and higher IL-10 secretion as compared with the non Tg littermates, indicating first a lack of T-cell anergy, and secondly, a shift from an inflammatory to a suppressogenic T-cell response. Low IFN-γ response in the NOD/DR4 Tg mice was attributed to a low number of CD8 T-cells secreting IFN-γ, and up regulation of Foxp3 expression in stimulated CD8 T-cells. Foxp3 can lower the IFN-γ synthesis by suppression of T-bet transcription required for IFN-γ synthesis (49; 71; 103). On the other hand, increased IL-10 secretion upon stimulation may be explained by an overall increased pool of CD4 T-cells observed in the NOD/DR4 Tg mice.

The NOD/DR4 Tg mice expressed an aberrant TCR Vβ repertoire, as reported for some HLA transgenic mice including the parental C57BL/6 (Abb KO), HLA-DRA1-DRB1*0401/I-$E^d_{\alpha 2\beta 2}$ Tg mouse (44) used to generate the NOD/DR4 Tg mouse. It has been suggested that such aberrations are linked to endogenous mouse mammary tumor

viruses (MMTV) harbored in the C57BL/6 mouse (MMTV-8, -9, and -17) (56), that may bind to the IE constant domains and preferentially delete T-cells expressing certain TCR Vβ families (88). First, no strong T1D-TCR Vβ correlations have been reported in the NOD mouse and humans, and thus the MMTV hypothesis may not explain the T1D resistance in NOD/DR4 Tg mice. Second, the MMTV hypothesis cannot explain the lack of an aberrant TCR Vβ repertoire in IL-10^+ TR-1 progenitors as compared with the $CD3^+$ T-cell pool, and why the TCR Vβ aberrations in $Foxp3^+$ Treg pool are not identical to those detected in the $CD3^+$ T-cell pool.

In contrast to the phenotypic and functional alterations in the T-cell compartment, the antigen-specific B-cell response in the NOD/DR4 Tg mice such as that to influenza virus immunization tested in this study, remained unaltered. This was somehow expected, since the primary anti-flu B-cell response occurs mainly in a T-cell independent manner (13). In summary, this study revealed regulatory effects of human *HLA-DR*0401* allele that can shape a T-cell compartment unable to uphold the T1D development, and it strongly suggests that T1D abrogation requires a simultaneous suppression of more than one T-cell subset. Similar HLA-DR*0401 regulatory events like in the NOD/DR4 Tg mice may occur in humans, but detection would be a hard task due to co-expression with other alleles. For this reason, the HLA transgenic mice can provide valuable insights on the role of individual HLA alleles in autoimmune disorders that are technically unattainable in humans.

Competing interests: The authors have declared that no competing interests exist.

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FIGURES AND FIGURE LEGENDS

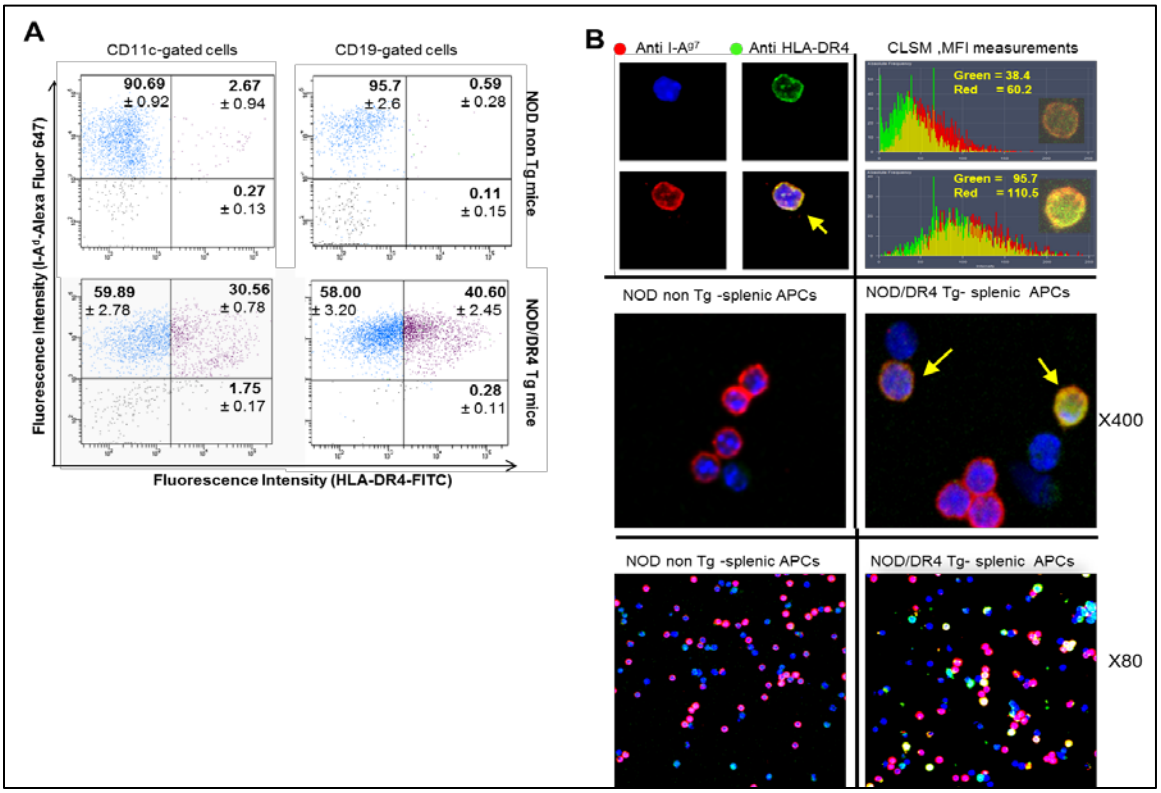


Figure 2.1 HLA-DR4 expression by APCs in the NOD/DR4 Tg mice.

Panel A, FACS analysis for HLA-DR4 and I-A^{g7} expression on splenic CD11c^{+} dendritic cells and CD19^{+} B-cells from NOD non Tg mice (n=4) and their NOD/DR4 Tg littermates (n=5). Cells were triple-stained with anti-mouse I-A^{d}-Alexa Fluor, anti-human HLA-DR-FITC, and anti-mouse CD11c-PE or anti-mouse CD19-PE conjugates. (Of note, the anti-I-A^{d} Ab recognizes the I-A^{g7} molecules which have only a replaced Asparagine amino acid with a Serine amino acid at position 57 in the β-chain). Some 200,000 cell events were acquired for individual mice in each group. Shown is the % mean ± SD values for positive cells in each group of mice. *Panel B*, CLSM single-cell analysis of I-A^{g7} and HLA-DR*0401 expression on enriched population of splenic APCs collected from 2 month-old NOD/DR4 Tg mice and NOD non Tg littermates; u*pper left*

panel, single-color stained APCs for nuclei with DAPI (blue color), anti-I-A^d Ab-Alexa Fluor 647 conjugate (red), anti-HLA-DR Ab-FITC conjugate (green). Shown is the I-A^{g7} and HLA-DR*0401 co-expression by double staining with anti-I-A^d and anti-HLA-DR Ab-dye conjugates (merged orange color, yellow arrow); *upper right panel*, MFI measurements based on the average color intensity of I-A^{g7} and HLA-DR4 stained molecules (n=3 mice per group) among 200 scored positive cells per mouse and using a ZEISS ZEN 2009 analysis software. Shown are two representative cells (yellow arrows, right lower panel) from one mouse in each group expressing different cell density of I-A^{g7} (red color histogram) and HLA-DR4 molecules (green color histogram). MFI absolute values for each color are shown in each quadrant, where X axis indicates the fluorescence intensity, and Y axis indicates the absolute frequency of pixels; *middle left panel*, group of splenocytes from a NOD non Tg mouse expressing only I-A^{g7} (red color), and *middle right panel*, group of splenocytes from a NOD/DR4 Tg littermate expressing both I-A^{g7} and HLA-DR4 molecules (merged, orange color). Of note, some cells staining only in blue (DAPI) in each group of mice indicates the lack of I-A^{g7} or HLA-DR4 expression on the surface; *lower panels*, large number of APCs stained with the same Ab-dye conjugates like in upper panels for a representative NOD non Tg mouse (*lower left panel*) and a NOD/DR4 Tg mouse. Of note, very few APCs (2-3%) express only HLA-DR4 molecules in the NOD/DR4 Tg mouse (lower *right panel*, green color).

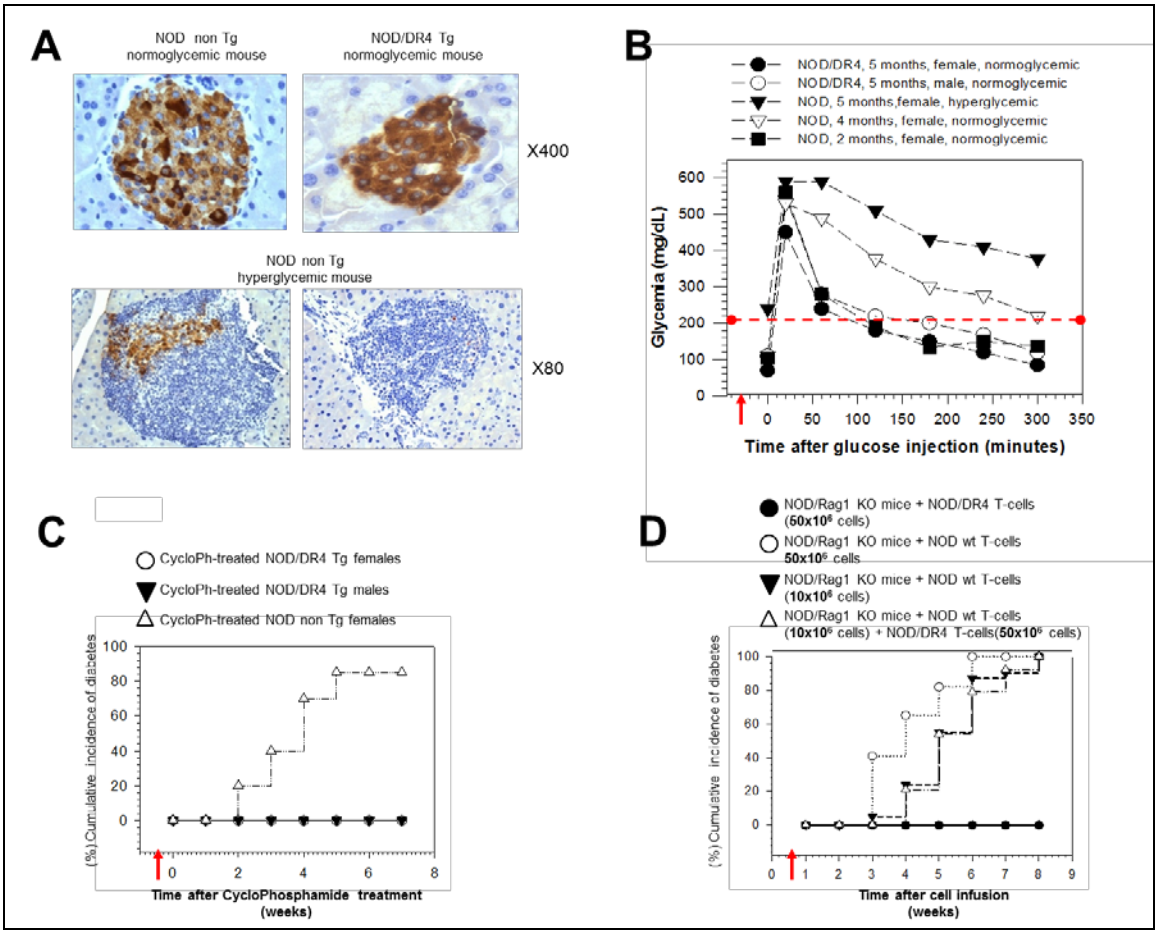


Figure 2.2 Resistance of NOD/DR4 Tg mice to T1D.

Panel A, a representative pancreatic β-islet from a NOD/DR4 Tg mouse and a non Tg littermate after staining with a rabbit anti-Insulin Ab-HRP conjugate to reveal the intra-islet granules of insulin (brown color), and over stained with HE to reveal the lymphocyte infiltration (blue color). *Panel B*, glucose tolerance test for adult NOD/DR4 Tg mice and NOD non Tg littermates' females and males as described. Glycemia was monitored every other hour after glucose load. Red arrow indicates the time of glucose load, and the red dotted line indicates the upper level of normoglycemia established in a cohort of 2-6 month-old NOD non Tg mice (n=25 mice). *Panel C*, 2 month-old NOD non Tg mice (n=7) and NOD/DR4 Tg littermates (n=7) were treated with Cyclophosphamide (CycloPh) as described, and the cumulative incidence of hyperglycemia (Y-axis) was

monitored weekly after the last injection. Red arrow (X-axis) indicates the time of last CycloPh injection. *Panel D*, 2 month-old NOD/Rag1 KO mice (n=7 mice/group) received i.p. splenic T-cells from hyperglycemic NOD wt mice in a mixture or not with splenic T-cells from a pool of 4 month-old euglycemic NOD/DR4 Tg mice, and glycemia was monitored on a weekly basis. Red arrow on X-axis indicates the time of cell infusion or co-infusion.

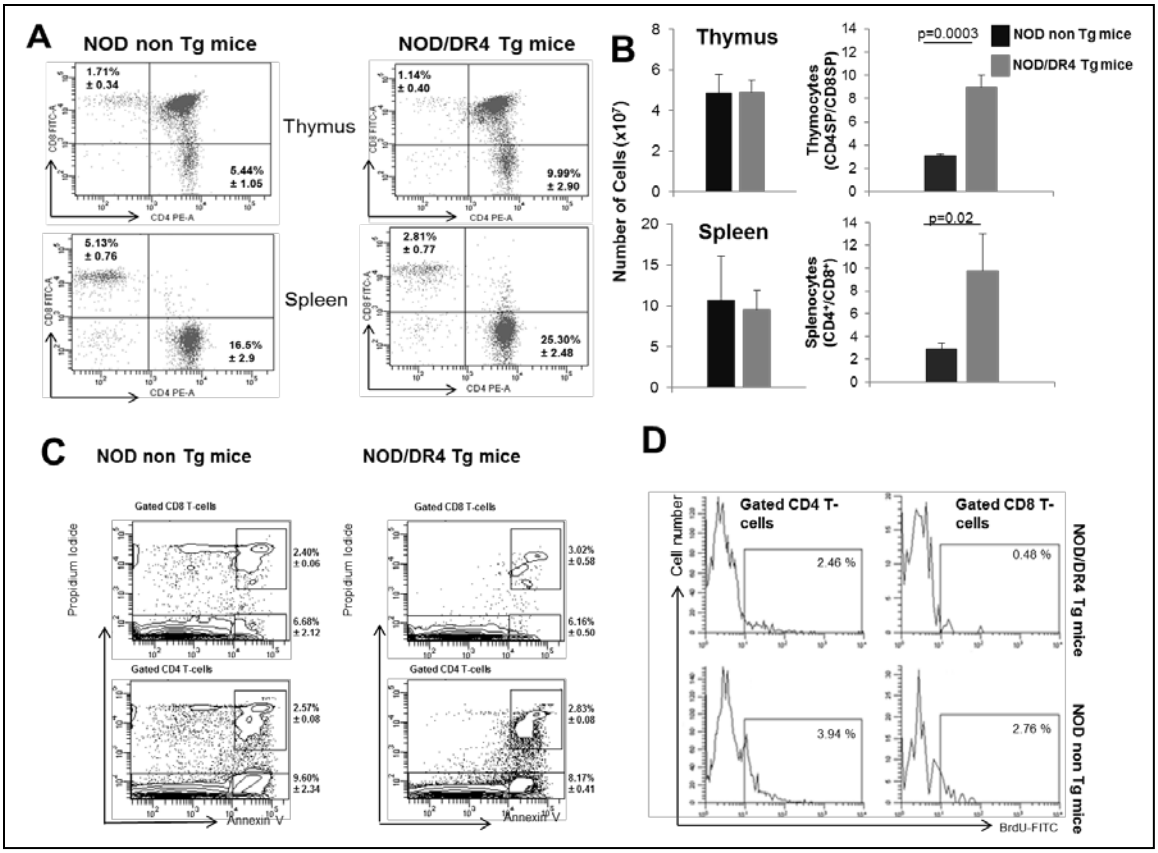


Figure 2.3 Frequency and phenotype of thymic and splenic CD4 and CD8 T-cells populations and their homeostatic rates in NOD/DR4 Tg mice and non Tg littermates.

Panel A, single-cell suspensions from the thymus or spleen of individual 2 month-old, female NOD/DR4 Tg mice (n=6) and NOD non Tg mice (n=6) double stained with CD4 Ab-PE and CD8 Ab-FITC conjugates (Invitrogen) analyzed by FACS. *Panel B*, Total cell numbers in the spleen and thymus were comparable in both groups of mice (n=6) (left). CD4/CD8 T cell ratios were significantly higher in the NOD/DR4 Tg mice compared to their NOD non Tg littermates (right). *Panel C*, spleen cells from the same mice in panel A were co-stained with CD4 Ab-PercP-Cy5.5 or CD8 Ab-Alexa Fluor 647, and over stained with Annexin V-FITC (Abcam) and Propidium Iodide (PI). Shown are gated CD4 and CD8 cells undergoing early apoptosis (low-right quadrant in each

histogram) and apoptotic cells (upper-right quadrant in each histogram). *Panel D*, single-cell suspensions from the spleen of individual 2 month-old, female NOD/DR4 Tg mice and NOD non Tg mice 1 hour after BrdU injection and analyzed for the rate of proliferation as described. Cells collected post-injection were stained with CD4 Ab-PercP-Cy5.5 or CD8 Ab-Alexa Fluor 647 and analyzed in individual mice in each group for the percent of cells that uptake BrdU.

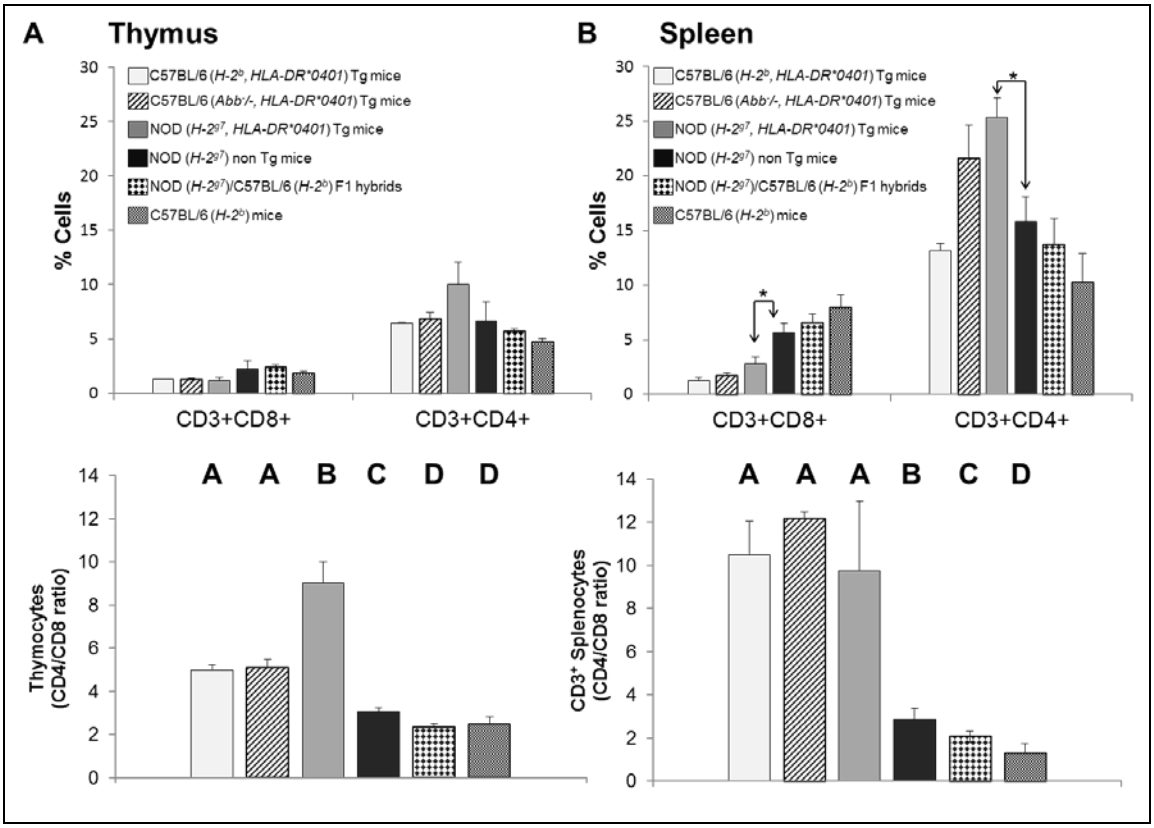


Figure 2.4 Effect of HLA-DR4 transgenic expression on the CD4 and CD8 T-cell frequency and ratio in the thymus and spleen of *H-2*^{g7} and *H-2*^{b} mice.

Panel A, single-cell suspensions (10^6 cells) from the thymus of 2 month-old C57BL/6 (*H-2*^{b}, *HLA-DR4*) Tg mice, or C57BL/6 (*Abb*$^{-/-}$, *HLA-DR4*) Tg mice, or NOD (*H-2*^{g7}, *HLA-DR4*) Tg mice, or NOD (*H-2*^{g7}) non Tg mice, or NOD (*H-2*^{g7})/C57BL/6 (*H-2*^{b}) F1 hybrid mice (4-6 mice/group) were individually co-stained with CD3 Ab-PE and CD4 Ab-PerCP (or CD8 Ab-FITC) conjugates Upper panel indicates the CD3-gated CD4^{+} and CD8^{+} T-cells frequency measured by FACS in individual mice from each group, and lower panel the corresponding CD4/CD8 T-cell ratios . *Panel B*, single cell suspensions (10^6 cells) from spleen from the same mice analyzed as in panel A. Upper panel shows the mean percentage ±SD of CD4 and CD8 T-cells in each group of mice and the significant difference for these cell populations in the spleen of NOD (*H-2*^{g7}, *HLA-DR*0401*) Tg mice and NOD (*H-2*^{g7}) non Tg mice (CD8, **p*=0.035; CD4,

*p=0.023). Of note, the HLA-DR4 expression altered significantly the CD4/CD8 ratio in the *H-2*^{g7} and *H-2*^{b} backgrounds. Lower panel shows the corresponding CD4/CD8 ratios between each groups described above. Non paired capital letters on the top of each column indicate the significant difference ($p<0.005$, t-test) between groups of mice, and paired capital letters indicate no statistical significance.

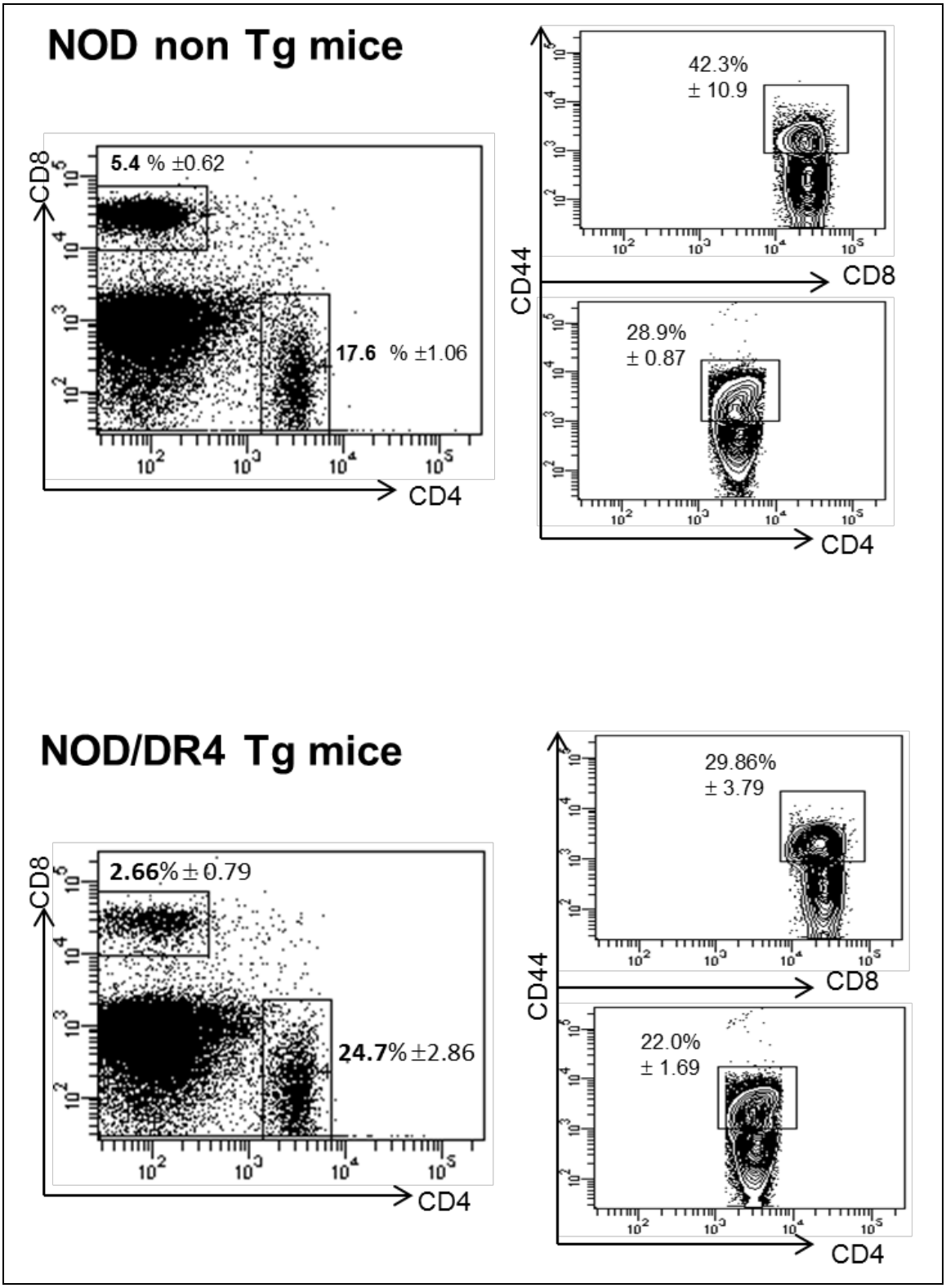


Figure 2.5 Frequency of T memory cells in NOD/DR4 Tg mice and non Tg littermates.

Single-cell suspensions from the spleen of individual 2 month-old, female NOD/DR4 Tg mice (n=4) and NOD non Tg mice (n=4) were triple stained with CD4 Ab-PercP-Cy5.5, CD8 Ab-Alexa Fluor 647, and CD44 Ab-PE. Quadrants marking the 10^3-10^4 fluorescence intensity for CD44 staining show the mean ± SD percent of memory ($CD44^{high}$) $CD4^+$ and $CD8^+$ T-cells.

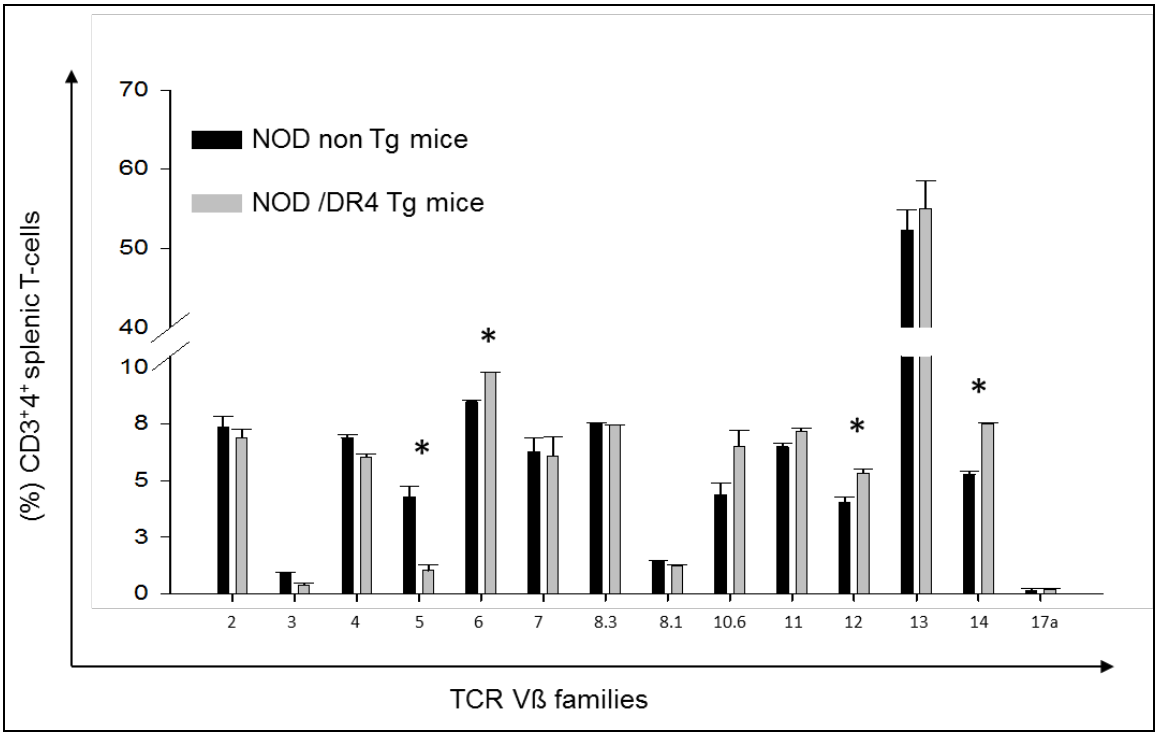


Figure 2.6 TCR Vβ repertoire in CD4 T-cells from NOD/DR4 Tg mice and non Tg littermates.

Single-cell suspensions of splenocytes from 2 month-old NOD non Tg mice (n=5) and NOD/DR4 Tg littermates (n=5) were co-stained with CD3-PE Ab, CD4 Ab-PercP-Cy5.5, and TCR Vβs Ab-FITC conjugates (Bioscience, Palo Alta, CA), and analyzed by FACS for the mean ± SD percent for each TCR Vβ family in individual mice from each group. Stars indicate significant differences (*$p \leq 0.05$) between the group of mice. Of note, Vβ-17a family has not been detected in NOD/DR4 Tg mice and their non Tg littermates.

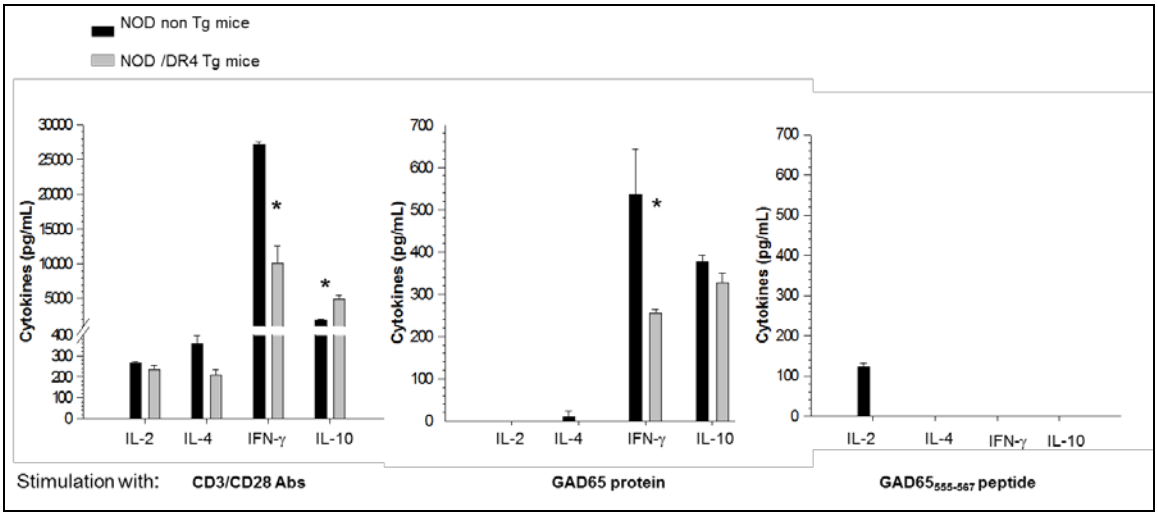


Figure 2.7 T-cell responses in NOD/DR4 Tg mice and NOD non Tg littermates.

10^6 splenocytes from individual 2 month-old NOD/DR4 Tg mice and their NOD non Tg littermates (n=4/group) were stimulated *in vitro* with CD3 and CD28 mAbs (2 μg mAb each) (*left panel*), or recombinant mouse GAD65 protein (10 μg/10^6 cells, *middle panel*), or synthetic $GAD65_{555-567}$ diabetogenic peptide (20 μg/10^6 cells, *right panel*), and the cytokines secreted in cell culture supernatants were measured by Luminex. Stimulation assays for IL-2 measurements were carried out for 24 h and for other cytokines for 72 h. Shown are the significant *p values between the group of mice (IFN-γ in the left panel (*p=0.03) and in the middle panel (*p=0.02), and for IL-10 in the left panel (*p=0.041).

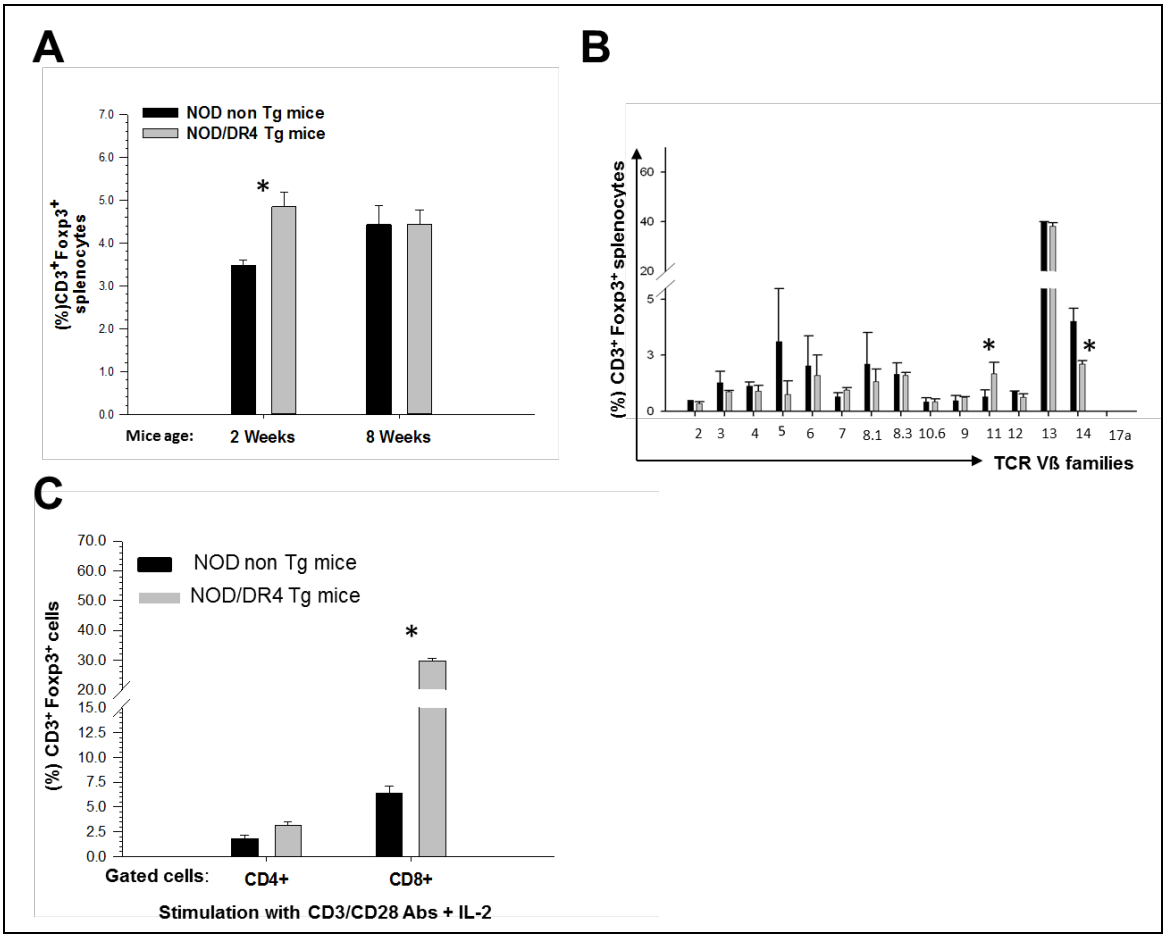


Figure 2.8 Frequency of Foxp3 Treg cells and TCR Vβ families expressing Foxp3 in the NOD/DR4 Tg mice and NOD non Tg littermates.

Panel A, splenocytes (10^6 cells) from 2 and 8 week-old NOD/DR4 Tg mice (n=7) and NOD non Tg littermates (n=5) were co-stained with CD3-PerCP and Foxp3 Ab-FITC conjugates, and cell frequency of Foxp3 expressing cells was measured by FACS. Shown is a significant difference between the mouse groups at 2 weeks of age (**p*=0.027). *Panel B*, splenocytes (10^6 cells) from 8 week-old NOD non Tg mice (n=4) and NOD/DR4 Tg littermates (n=5) were co-stained with CD3-PerCP, Foxp3 Ab-PE, and TCR Vβs Ab-FITC conjugates, and the frequency of $CD3^+Foxp3^+$ cells in each Vβ family from individual mice in each group was measured by FACS. Shown are significant differences between the groups for Vβ11 (**p*=0.02) and Vβ14 (**p*=0.03). Of

note, Vβ-17a family has not been detected in NOD/DR4 Tg mice and their non Tg littermates. *Panel C*, splenocytes (10^6) from individual 8 week-old NOD non Tg mice (n=5) and NOD/DR4 Tg littermates (n=5) were stimulated *in vitro* for 3 days with CD3 and CD28 Abs (2 μg each) in the presence of rIL-2 (100 mU), and then co-stained with CD3 Ab-PerCP, CD4 Ab-APC (or CD8 Ab-APC), and Foxp3 Ab-FITC. Shown is the mean ± SD percentage of $CD3^+4^+Foxp3^+$ cells and $CD3^+8^+Foxp3^+$ cells measured by FACS in each group of mice, and the significant difference for $CD3^+8^+Foxp3^+$ cells between the group of mice (*p=$3.8x10^{-6}$).

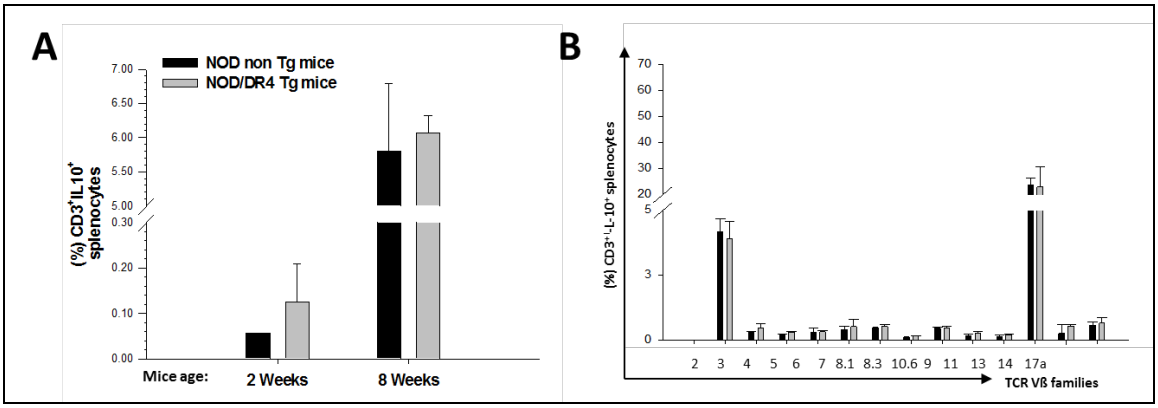


Figure 2.9 Frequency of IL-10$^+$ TR-1 cells and TCR Vβ families expressing IL-10 in the NOD/DR4 Tg mice and NOD non Tg littermates.

Panel A, splenocytes (10^6 cells) from individual 2 and 8 week-old NOD non Tg mice (n=4) and NOD/DR4 Tg littermates (n=5) were first stimulated *in vitro* for 2 days with CD3/CD28 mAbs (2.5 μg mAb each/10^6 cells). Stimulated cells were treated for the last 4 h with monensin (Golgi Stop, # 554715) according to the manufacturer instructions (BD Biosciences, San Diego, CA), and then intracellularly stained with IL-10 Ab-APC conjugate and surfaced stained with CD3 Ab-FITC conjugate. Shown is the mean ± SD percentage of IL-10 positive T-cells analyzed by FACS for in individual mice from each group. *Panel B*, splenocytes (10^6 cells) from 8 week-old NOD non Tg mice (n=4) and NOD/DR4 Tg littermates (n=5) were stimulated as described in panel A, and co-stained with CD3-PerCP, IL-10 Ab-APC, and TCR Vβσ Ab-FITC conjugates. Frequency of CD3$^+$IL-10$^+$ cells in each Vβ family was measured by FACS in individual mice from each group. Of note, there are no differences among the CD3$^+$IL-10$^+$ Vβ families between the two groups of mice.

SUPPLEMENTAL FIGURES:

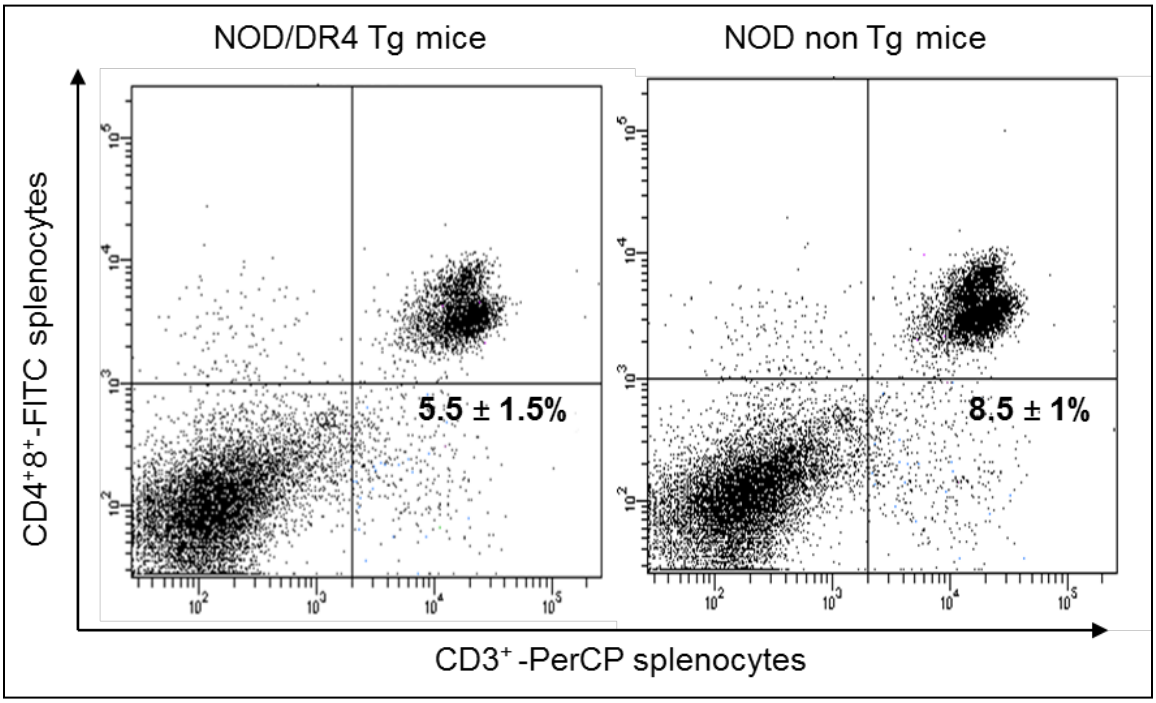
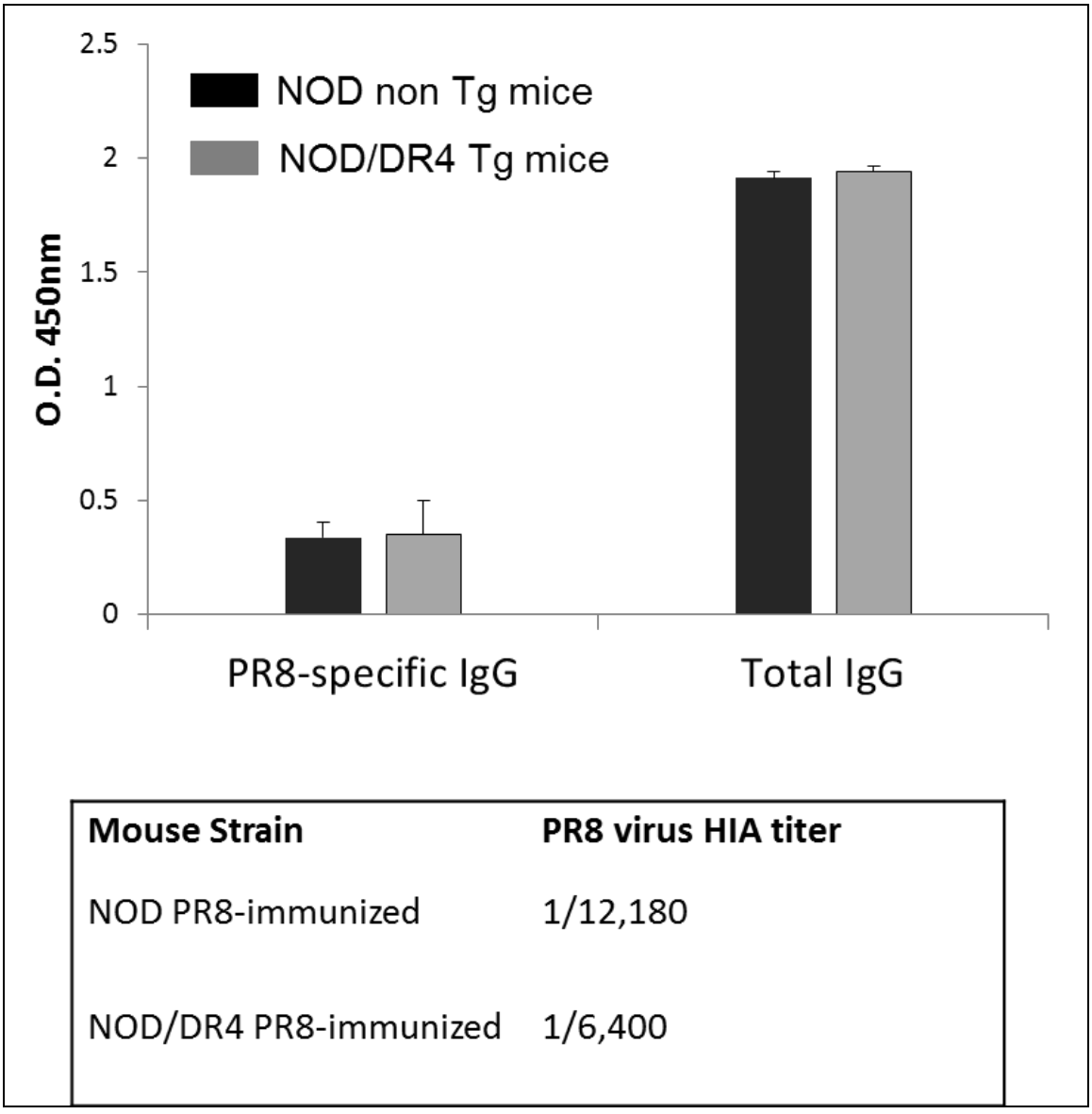


Figure 2.S1 Frequency of $CD3^{+}4^{-}8^{-}$ double negative T regulatory cells in NOD/DR4 Tg mice and NOD non Tg littermates.

Splenocytes (10^{6}) from individual 2 week-old NOD non Tg mice (n=5) and NOD/DR4 Tg littermates (n=6) were stained for CD4, CD8 and CD3 markers, and the mean ± SD percentage of $CD3^{+}4^{-}8^{-}$ double negative T-cells (DNCD3 cells) was measured by FACS.



Mouse Strain	PR8 virus HIA titer
NOD PR8-immunized	1/12,180
NOD/DR4 PR8-immunized	1/6,400

Figure 2.S2 B-cell response in NOD/DR4 Tg mice and NOD non Tg littermates to influenza virus.

NOD/DR4 Tg mice and their NOD non Tg littermates (n=5/group) were immunized with UV-inactivated influenza PR8/A/34 virus as described. Sera was collected and measured 21 days later for the immunoglobulin and virus-specific antibody titers by ELISA (*panel A*) and for the neutralizing anti-influenza Ab titers by HIA (*panel B*), as described.

Chapter 3: Long-term silencing of autoimmune diabetes and improved life expectancy by a soluble pHLA-DR4 chimera in newly-humanized NOD/DR4/B7 mice

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ABSTRACT

Several human MHC class II (HLA) molecules are strongly associated with high incidence of autoimmune diseases including type 1 diabetes (T1D). The HLA-humanized mice may thus represent valuable tools to test HLA-based vaccines and therapeutics for human autoimmune diseases. Herein, we have tested the therapeutic potential of a soluble HLA-DR4-GAD65$_{271-280}$ chimera of human use in a newly-generated NOD/DR4/B7 double transgenic (dTg) mouse that develops spontaneously an accelerated T1D early in life and regardless the gender. The NOD/DR4/B7 dTg mice were generated by a two-step crossing protocol, and they expressed HLA-DR*0401 molecules on 20% of antigen presenting cells, human B7 molecules in the pancreas, and HLA-DR4/GAD65-specific T-

cells in the blood. Some 75% of pre-diabetic NOD/DR4/B7 dTg mice treated with HLA-DR4-GAD65$_{271-280}$ chimera (huNOD-GAD65 reagent) remained euglycemic and showed stabilized pancreatic insulitis 6 months after treatment. The 25% non-responder mice that developed hyperglycemia also survived 3-4 months longer than their untreated littermates. T1D prevention by this reagent occurred by a Th2/TR-1 polarization in the pancreas. This study strongly suggests that the use of soluble HLA-peptide reagents to suppress/stabilize the T1D progression and to extend the life expectancy in the absence of side effects is an efficient and safe therapeutic approach.

Keywords: Human HLA-peptide chimera, Humanized NOD mouse, T1D stabilization.

INTRODUCTION

Type 1 diabetes mellitus (T1D) is an organ-specific autoimmune disease induced by a polyclonal population of self-reactive T-cells that lead to the destruction of insulin-secreting pancreatic ß-cells (39). High incidence of T1D is strongly associated with the expression of particular human MHC class I and II alleles (i.e., HLA-DR4, HLA-DQ8, HLA-A2.1) and murine MHC class II (I-A^{g7}) in the NOD mice (30; 65). Some 60% of the T1D patients in USA express HLA-DR4 alleles (83).

Several non-antigen specific immunosuppressive attempts to suppress T1D progression showed minimal beneficial effects in experimental conditions or clinical trials (25; 61; 67). Some of these approaches also raised safety concerns like induction of systemic immune suppression (65) or pancreatic toxicity (79). We and others reported that a new class of antigen-specific reagents, namely soluble peptide-MHC class II chimeras (DEF reagents, “Diabetes Eliminating Factor”) can delay the pre-clinical stage of diabetes, and more importantly reverse the early T1D onset in mouse models in the

absence of side-effects and more efficiently than the synthetic peptide preparations (16; 59; 60; 66). A murine DEF reagent was also able to protect grafted pancreatic islets against the re-emerging diabetes (17). DEF reagents have a 2 to 3-day life-span in vivo (82), do not require adjuvant to reach the therapeutic effect, and are devoid of side effects (16; 17; 60; 82). On a molar basis, a soluble DEF dimer can deliver ≈1,000-times more tolerogenic peptide than the APC-expressing MHC class II molecules can naturally load in vivo (109). DEF anti-diabetogenicity was shown to rely mostly on the induction of IL-4-secreting Th2 cells and IL-10-secreting TR-1 suppressor cells in the pancreas (16; 17; 109).

We previously showed that a soluble dimeric HLA-DR*0401-GAD65$_{271-285}$ chimera (huDEF-GAD65 reagent) of human use can induce IL-10-secreting TR-1 cells by GAD65$_{271-285}$–specific T-cells in the peripheral blood lymphocytes of diabetic patients (83). To test the therapeutic efficacy of this reagent, we have generated a suitable humanized NOD strain expressing on the APCs the human MHC class II HLA-DR*0401 molecules under the murine MHC class II *I-E*^{d} gene promoter, and at the same time the human B7.1 (CD80) costimulatory molecule under the rat insulin promoter in the pancreatic β-islets (NOD/DR4/B7 dTg mouse). While the NOD/DR4 Tg parental mouse used to generate our NOD/DR4/B7 dTg mouse does not develop diabetes (81), the NOD/DR4/B7 dTg mice develop an aggravated, spontaneous disease early in life and regardless the gender. These humanized NOD/DR4/B7 dTg mice were used in this study to test the preventive capacity of hu DEF-GAD65 reagent.

RESULTS AND DISCUSSION

The humanized NOD/DR4/B7 dTg mouse is a suitable model for testing the therapeutic potential of hu DEF-GAD65 reagent.

The NOD wt mouse is the closest model for human T1D (30). Herein, we tested the therapeutic effect of a hu HLA-DR4-$GAD65_{271-285}$ chimera of human use in a newly-generated, humanized NOD strain expressing the human MHC class II HLA-DR*0401 molecule on APCs under the I-E^d gene promoter and human B7.1 costimulatory molecule under the rat insulin promoter in pancreas (NOD/DR4/B7 dTg mouse). The soluble dimeric hu HLA-DR4-$GAD65_{271-285}$ chimera is referred thereafter as to huDEF-GAD65 reagent.

Full recovery of the NOD diabetogenic background in the parental NOD/DR4 Tg mouse used to generate the NOD/DR4/B7 dTg mouse was confirmed by PCR and microsatellite analysis (**Fig. 3.1A**). The HLA-DR*0401 requirement for the NOD/DR4/B7 dTg mouse was to present the $GAD65_{271-285}$ self-peptide to CD4 T-cells, which in turn could be targeted by the hu DEF-$GAD65_{271-285}$ reagent. The hu DEF-$GAD65_{271-285}$ reagent has been previously shown to recognize and score by FACS the human $GAD65_{271-285}$-specific CD4 T-cells from HLA-DR*0401^+ diabetic patients (83). FACS analysis confirmed the specific binding of hu DEF-$GAD65_{271-285}$ reagent to the hu HLA-DR*0401 transgenic molecule on more than 20% of splenic cells (**Fig. 3.1B**), as well as the presence of $GAD65_{271-285}$-specific CD4 T-cells in the spleen of NOD/DR4/B7 dTg mice (**Fig. 3.1C**). On the other hand, pancreatic B7.1 costimulation of diabetogenic T-cells was previously shown to accelerate the T1D onset and to aggravate the disease progression in mouse models (18). PCR and immunohistochemical analyses confirmed the hu B7.1 expression in the pancreatic islets of NOD/DR4/B7 dTg mouse (**Fig. 3.1D**).

This mouse develops an aggressive, spontaneous diabetes by 3 to 4 months after birth and regardless the gender (**Fig. 3.2A**). Together, these data confirmed the suitability of our humanized NOD/DR4/B7 dTg mouse for testing the therapeutic potential of hu DEF-$GAD65_{271-285}$ reagent.

Human DEF-GAD65 reagent delays the T1D onset in NOD/DR4/B7 dTg mice

The rationale of using a different GAD65 peptide sequences expressed by human and murine DEF-GAD65 reagents is that the $GAD65_{217-230}$ peptide expressed by the murine DEF-GAD65 reagent is recognized only by the MHC class II ($I\text{-}A^{g7}$) in NOD mouse, whereas the $GAD65_{271-280}$ peptide expressed by the human DEF-GAD65 reagent is recognized only by the HLA-DR4 molecules in humans.

Treatment with pre-diabetic NOD/DR4/B7 dTg mice with hu DEF-GAD65 reagent in saline or saline alone (control group) was initiated 3 months after birth when mice show an euglycemic status and light pancreatic insulitis. Some 75% of the NOD/DR4/B7 dTg mice (n=14) treated i.p. twice a week with 8 small doses (10 μg/dose) of hu DEF-GAD65 reagent did not develop hyperglycemia for 6 months after treatment (p=0.009) (**Fig. 3.2A**). In contrast, 75% of the NOD/DR4/B7 dTg littermates in the control group (n=9) developed hyperglycemia shortly after the last i.p. injection of saline alone (p=0.01). These data demonstrated that, like the murine DEF-GAD65 reagent (16; 60), the human DEF-GAD65 reagent exerted similar therapeutic effects in the humanized NOD/DR4/B7 dTg mouse model.

Although our present focus was to test the T1D preventive ability of hu DEF-GAD65 reagent, this study suggests the likability of this reagent to reverse the early T1D onset. Thus, among the hu DEF-GAD65-treated mice with sustained euglycemic status, 1

mouse developed hyperglycemia 1 month after the last injection. This mouse responded well to an additional i.p. injection of 20 μg of DEF-GAD65 reagent when hyperglycemia scored 240 mg/dL and 270 mg/dL, as it returned next day to a stable euglycemic status for another 3 months (**Fig. 3.2B**). In contrast, several mice in the saline-treated group (control group) that have been also treated with a single dose of 20 μg hu DEF-GAD65 reagent when reaching higher sugar levels than 400 mg/dL, did not respond to the treatment. This led to the conclusion that, like previously shown in animal models treated with other murine DEF-like reagents, the hu DEF-GAD65 reagent may not be able to reverse the disease when hyperglycemia reaches higher levels than 300 mg/dL. Confirmatory therapeutic strategies using different protocols of immunization will be further required to determine if indeed the hu DEF-GAD65 reagent is beneficial in the late stages of disease.

Human DEF-GAD65 treatment improves the rate of survival in diabetic NOD/DR4/B7 dTg mice

Although ≈25% of NOD/DR4/B7 dTg mice did not respond to hu DEF-GAD65 therapy, their overall life expectancy was significantly increased. Two of the hyperglycemic non responders lived up to 4 more months after the interruption of treatment. Also, most of the hyperglycemic NOD/DR4/B7 dTg mice in the saline-treated group (control group) survived for 2 to 3 more months longer, and did not lose weight (data not shown) when injected i.p. with a single dose of 20 μg of hu DEF-GAD65 reagent (**Fig. 3.2B)**. This is a significantly long rate of survival as compared with the untouched hyperglycemic littermates that succumb within 2 to 3 weeks after the onset of hyperglycemia. The non-responder mice treated with a single dose of 20 μg hu DEF-

GAD65 reagent maintained their sugar levels below 550 mg/dL and showed no body weight loss (data not shown). The results indicated that, although in a hyperglycemic stage, the mice survived longer with a limited progression of hyperglycemia when treated with hu DEF-GAD65 reagent at a late stage of disease.

Human DEF-GAD65 reagent stabilizes the lymphocyte infiltration in the pancreas of NOD/DR4/B7 dTg mice

We have previously reported that mice treated with murine DEF reagents show a significantly reduced number of pancreatic β-islets with destructive intra-islet infiltration, but rather a protective type of peri-insulitis (16; 17; 60). Indeed, prolonged survival observed in the non-responder, hyperglycemic mice treated with hu DEF-GAD65 may be explained by the formation of a protective, stabilized peri-islet pancreatic infiltration, which in turn may provide a limited, but sufficient amount of insulin to stabilize glycemia.

The NOD/DR4/B7 dTg mice protected by hu DEF-GAD65 treatment showed a significantly increased number of peri-infiltrated β-islets ($p=0.047$), and a reduced number of intra-infiltrated β-islets ($p= 0.042$) than those in the control group (**Fig. 3.3A & 3.3B**). Although not significantly increased, the number of non-infiltrated β-islets in euglycemic mice protected by hu DEF-GAD65 treatment was higher than in hyperglycemic mice from the control group ($p=0.063$). This is because the overall number of infiltrated β-islets (intra- and per-infiltrated β–islets together) in both groups was similar, with the only difference that the ratio between the peri- *vs*. intra-infiltrated β–islets was higher in the euglycemic treated mice (ratio= 4:1) than in hyperglycemic mice from the control group (ratio= 1:3.5) (**Fig. 3.3B**). The results indicated that, likewise

the murine DEF-GAD65 reagent (16; 17; 60), the human DEF-GAD65 reagent was able to stabilize the pancreatic insulitis.

The therapeutic effects of hu DEF-GAD65 reagent rely on Th2/TR-1 polarization in pancreas

We have previously reported that the mechanism of T1D protection by murine DEF reagents relies on differentiation and expansion of IL-4^+ Th2 cells and IL-10-secreting TR-1 suppressor cells in the pancreas (17; 18; 60). Also, the hu DEF-GAD65 reagent induced a population of IL-4^+ Th2 cells and IL-10-secreting TR-1 cells in the lymphocyte cultures of diabetic patients expressing HLA-DR*0401 molecules (83). Herein, we tested whether the mechanism underlying T1D protection by the hu DEF-GAD65 reagent in NOD/DR4/B7 dTg mice relies also on Th2/TR-1 polarization. The Th2 response in stimulated cell cultures from the pancreatic lymph nodes of NOD/DR4/B7 dTg mice treated or not with hu DEF-GAD65 reagent was estimated based on IL-4 secretion, the TR-1 response based on IL-10 secretion, and the Th1 based on IFN-γ secretion.

CD4 T-cells from the pancreatic lymph nodes (pLN CD4 T-cells) mirror the phenotypic and functional profile of pancreatic infiltrated T-cells in hyperglycemic mice (15). The pLN CD4 T-cells from treated euglycemic mice that were stimulated *in vitro* with hu DEF-GAD65 reagent 6 months after treatment interruption showed a significant increase in IL-4 secretion ($p=0.0001$) and IL-10 secretion ($p=0.018$), whilst the IFN-γ secretion was drastically reduced ($p=4.5x10^{-5}$) as compared with the hyperglycemic mice in the control group. Also, the *in vitro* CD3 polyclonal stimulation of pLN CD4 T-cells from euglycemic treated mice showed a significantly reduced IFN-γ secretion ($p=0.039$)

and increased IL-10 secretion (p=0.014) as compared with those from the hyperglycemic control mice (**Fig. 3.3C**).

Together, these results showed that T1D prevention and stabilization in NOD/DR4/B7 dTg mice occurred in the context of Th2/TR-1 polarization of peripheral CD4 T-cells, much likely by a similar mechanism (single-epitope bystander suppression) described for the murine DEF-GAD65 reagent (60). This mechanism refers as to the ability of a single peptide-MHC class II reagent (like DEF) to suppress a polyclonal population of autoreactive T-cells of various peptide specificities through the stimulation/expansion of non-antigen-specific IL-10-secreting TR-1 suppressor cells at the site of inflammation (60). In summary, this work suggests that human DEF-like reagents targeting diabetogenic T cells in the absence of systemic immune suppression may represent an efficient antigen-specific approach that can overcome the limitations of conventional antigen-specific reagents for T1D therapy.

METHODS

The human DEF-GAD65 reagent.

Genetic engineering of human HLA-DR*0401-GAD65$_{271-285}$ soluble dimer (hu DEF-GAD65 reagent) has been previously described.(4) The hu DEF-GAD65 reagent is made of the extracellular domains of human HLA-DR*0401 (MHC class II) molecules on human Ig-Fcγ1 (hFcγ1) scaffold, and it expresses the human GAD65$_{271–285}$ peptide covalently-linked at the N terminus of β–chain. The hu DEF-GAD65 reagent was produced in baculovirus-infected insect SF9 cells and purified from the cell culture supernatant by affinity chromatography using an anti-human IgG1-Sepharose 4B column, as described (4; 83).

The humanized NOD/HLA-DR4/B7 dTg mouse.

The humanized NOD/DR4/B7 dTg strain was generated by a two-step crossing protocol. First, the C57BL/6 mice deficient for MHC class II molecules (H-2^{b+}, IA$\beta^{-/-}$ /IE$\alpha^{-/-}$) and transgenic for a human/murine chimeric HLA-DR4-IE molecule (HLA-DRA-$IE^{d}\alpha$/HLA-DRB1*0401-$IE^{d}\beta$) (Jackson Labs) (44) were backcrossed for 12 generations into the NOD diabetogenic background (IA^{g7+}, IE^{d}_{null}, H-2^{d+}) to generate the NOD-DR4 Tg strain (HLA-DRA-$IE^{d}\alpha$/HLA-DRB1*0401-$IE^{d}\beta^{+}$, IA^{g7+}, IE^{d}_{null}, H-2^{d+}). The NOD/DR4 Tg mice were next crossed with NOD/RAG$2^{-/-}$, hu B7.$1^{+/+}$ Tg mice (Taconic) to generate the NOD/DR4/B7 dTg mice used in this study (HLA-DRA-$IE^{d}\alpha$/HLA-DRB1*0401-$IE^{d}\beta^{+}$, huB7.1^{+}, IA^{g7+}, $IE^{d(null)}$, H-2^{d+}, Rag2^{+}). Selection of NOD/DR4/B7 dTg mice was carried out by PCR using specific primers for HLA-DR4 and hu B7.1 genes (**Fig. 3.1B**).

Therapeutic protocol.

Pre-diabetic (euglycemic) NOD/DR4/B7 dTg mice were injected intraperitoneal (i.p.) every other 4^{th} day with 8 doses of 10 μg of hu DEF-GAD65 reagent in saline (n=14 mice) or saline alone (control group, n=9 mice) every other 4^{th} day, starting 3 months after birth. Mice were followed weekly for the blood glucose levels using an Accu-Check glucose meter and glucose test strips (Roche). Non-responder hyperglycemic mice were also treated with a supplemental dose of 20 μg hu DEF-GAD65 reagent after the hyperglycemia onset, and monitored for the blood glucose levels and rate of survival. Experiments were carried out at USUHS under the MED-11-655 and MED-11-805 IACUC protocols according to the federal, local regulations, and to EU Directive 2010/63/EU.

Flow cytometry.

Single-cell suspension of splenocytes (10^6 cells) from individual mice were stained 30 min at 4°C for various cell surface markers using specific Ab-dye conjugates or their isotype controls. Some 10^4 to10^5 cell events were acquired using a LSR II Becton-Dickinson flow cytometry instrument equipped with the WINLIST analysis software (Verity, Topsham, ME, USA), or with a BD FACS DIVA software (BD Biosciences).

T-cell stimulation and cytokine assays.

Cells from the pancreatic lymph nodes of individual treated and saline-treated mice were harvested on 5μm strainers and stimulated *in vitro* for 1 and 4 days with CD3 mAb (#2C11 clone, ATCC, 2.5 μg Ab/10^6 cells/well) or hu DEF-GAD65 reagent (5 μg reagent/10^6 cells/well). Cell viability scored microscopically in trypan blue stained cells was higher than 90%. The supernatant from triplicates cell cultures was measured by Luminex assay for cytokine secretion. IL-2 measurements were carried out 1 day after stimulation, whereas other cytokines were measured 4 days after continuous stimulation.

Histology and Immunohistochemistry.

Pancreata from individual mice responsive or not to hu DEF-GAD65 treatment were analyzed for the degree and morphology of insulitis among 20-25 paraffin-embedded 5μm sections stained with Hematoxilin-Eosin (HE). Serial 5μm sections of pancreata were also stained with a rabbit anti-insulin Ab (Santa Cruz Biotech, Santa Cruz, CA) revealed by a goat anti-rabbit IgG-HRP conjugate (Southern Biotechnologies, Birmingham, AL) to estimate the extend of insulin secretion and intra-islet distribution of insulin granules.

Biostatistics.

Statistical significance for T1D incidence within the same group and between groups of hu DEF-GAD65-treated and saline-treated mice was carried out by Log-rank (Mantel-Cox) test, SPSS software version 21.0.0 (IBM Corporation 1 New Orchard Road Armonk, New York 10504-1722 United States). The *p* values less than 0.05 were considered significant. Individual variations in the number of infiltrated islets and morphology (protective peri-insulitis *vs*. destructive intra-islet insulitis) between hu DEF-GAD65-treated and saline-treated mice were analyzed by the Student's *t*-test and expressed as mean ± standard deviation (SD) at 99% interval of confidence.

Conflict of interest: The authors declare no actual or potential conflict of interest related to this work.

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FIGURES AND FIGURE LEGENDS

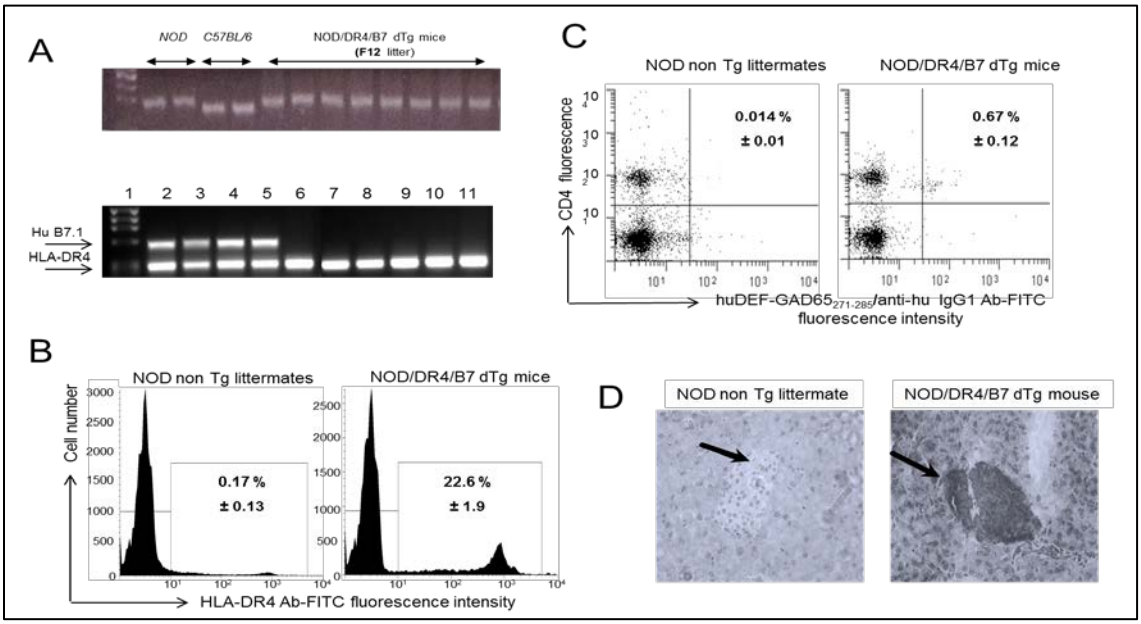

Figure 3.1 Immunologic characterization of humanized NOD/DR4/B7 dTg mice.

Panel A, microsatellite analysis of the NOD genetic background in the parental NOD/DR4 Tg strain (upper panel). Comparison between the genetic background in 2 representative NOD wt, C57BL/6 parental strains, and 8 out of 32 microsatelites in the F12 generation of parental NOD/DR4 Tg strain shows fully transfer of the NOD background in the parental NOD/DR4 Tg strain. Lower panel, identification of human HLA-DR*0401 and B7.1 (CD80) transgenes by PCR using specific primers (forward: GTTTCTTGGAGCAGGTTAAACA; reverse: CTGCACTGTGAA GCTCTCAC, and respectively: forward: GCTTACAACCTTTGGAG ACCCAG; reverse: CGTCACTTCAGCCAGGTG). Internal control PCR primers for DNA quantification were specific for mouse IgG3 gene (forward: ACAACAGCCCCATCTGTCTAT; reverse: GTGGGCTACGTTGCAGATGAC). Lane 1, DNA size markers; lanes 2-5, NOD mice expressing both the human HLA-DR*0401 and B7.1 transgenes; lanes 6-11, NOD/DR4 littermates lacking the hu B7.1 transgene.

Panel B, *e*xpression of HLA-DR4 molecules on splenic APCs from NOD/DR4/B7 dTg mice. Left panel, splenic cells stained with a rat IgG isotype control Ab-FITC conjugate; Right panel, splenic cells stained with a rat IgG anti-HLA-DR4-FITC conjugate. Shown are the mean ± SD values as determined in 4 NOD/DR4/B7 mice. ***Panel C***, FACS detection of $GAD65_{271-285}$-specific $CD4^+$ T-cells in the blood of NOD/DR4/B7 dTg mice. Left panel, the signal-to-noise background of the secondary anti-human IgG1-FITC conjugate; right panel, the mean frequency of $GAD65_{271-280}$-specific $CD4^+$ T cells ± SD measured in 4 NOD/DR4/B7 dTg mice in spleen cells double-stained with hu DEF-GAD65 reagent and revealed by a goat anti-human IgG1-FITC conjugate, and anti-mouse CD4 Ab-APC conjugate (clone #GK1.5, ATCC, BD PharMingen). ***Panel D***, immunohistochemical detection of human B7.1 (CD80) expression in the pancreatic β-islets of NOD/DR4/B7 dTg mice. Fresh pancreatic sections of 5μ in OCT from a NOD wt mouse (left panel) and NOD/DR4/B7dTg mouse (right panel) were stained with a rat IgG anti-human B7 molecule (BD PharMingen) and revealed by an anti-rat IgG-HRP conjugate (Jackson ImmunoResearch). Shown is the absence of B7 staining of a representative β-islet from a NOD wt mouse, and the positive B7 staining for a representative β-islet from a NOD/DR4/B7 dTg mouse. Dark arrows in each panel indicate the position of pancreatic β-islets.

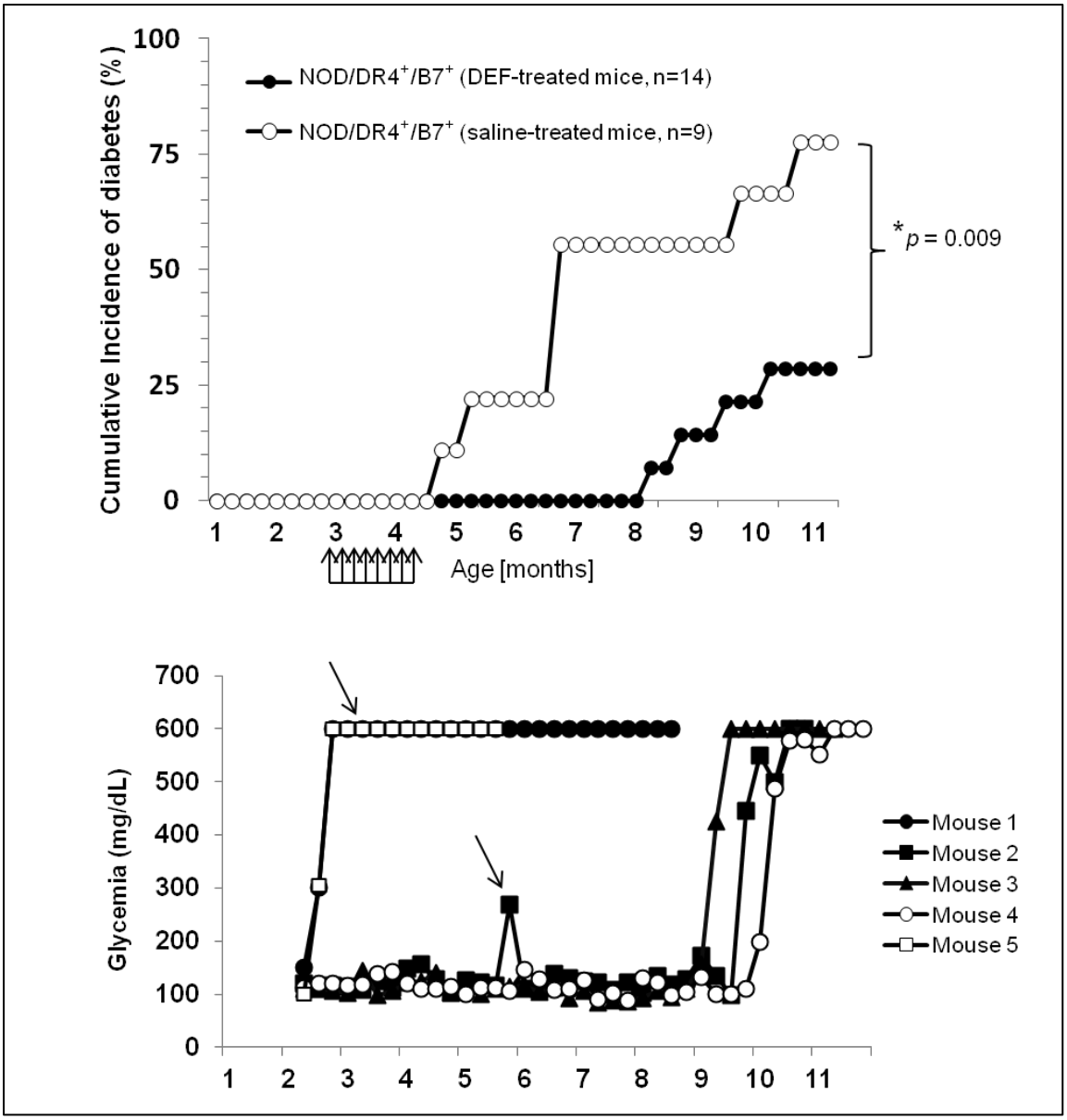

Figure 3.2 T1D incidence in the NOD/DR4/B7 dTg mice treated or not with hu DEF-GAD65 reagent.

Panel A, Pre-diabetic, 2.5 month-old NOD/DR4/B7 dTg mice were selected from 4 different litters (n=14) and treated i.p. with 8 doses of 10 μg of hu DEF-GAD 65 reagent in saline (n=14 mice) or with saline alone (control group, n=9 mice) every other 4th day. Glycemia was measured bi-weekly from the tail vein. Y axis indicates the cumulative incidence of hyperglycemia calculated as a percent of mice developing

hyperglycemia in each group at different time-points after treatment interruption. Grouped arrows on the X axis indicate the time-points and number of hu DEF-GAD65 i.p. injections. Shown is the significant relevance (* *p* value) between the two groups at the end of experiment. ***Panel B***, hyperglycemia values in hu DEF-GAD65 non responders (NOD/DR4/B7 dTg mice) in two representative mice (Mouse #1 and #3) with stabilized hyperglycemia after one single dose of 20 μg hu DEF-GAD65 as followed up for 8 more months after injection. Arrows indicate the time of supplemental hu DEF-GAD65 injection (at 6 months of age) that failed to reverse hyperglycemia in mice #1 and #3. Also shown is a NOD/DR4/B7 dTg mouse (mouse #2) treated with hu DEF-GAD65 reagent under the same regimen as in panel A, which developed mild hyperglycemia (250 and 270 mg/dL) some 1.5 months after treatment interruption. Arrow indicates the time-point (6 month of age) when mouse #2 received a supplemental 20 μg hu DEF-GAD65 i.p. injection that reversed hyperglycemia. Grouped arrows on the X axis indicate the time-points and number of hu DEF-GAD65 i.p. injections.

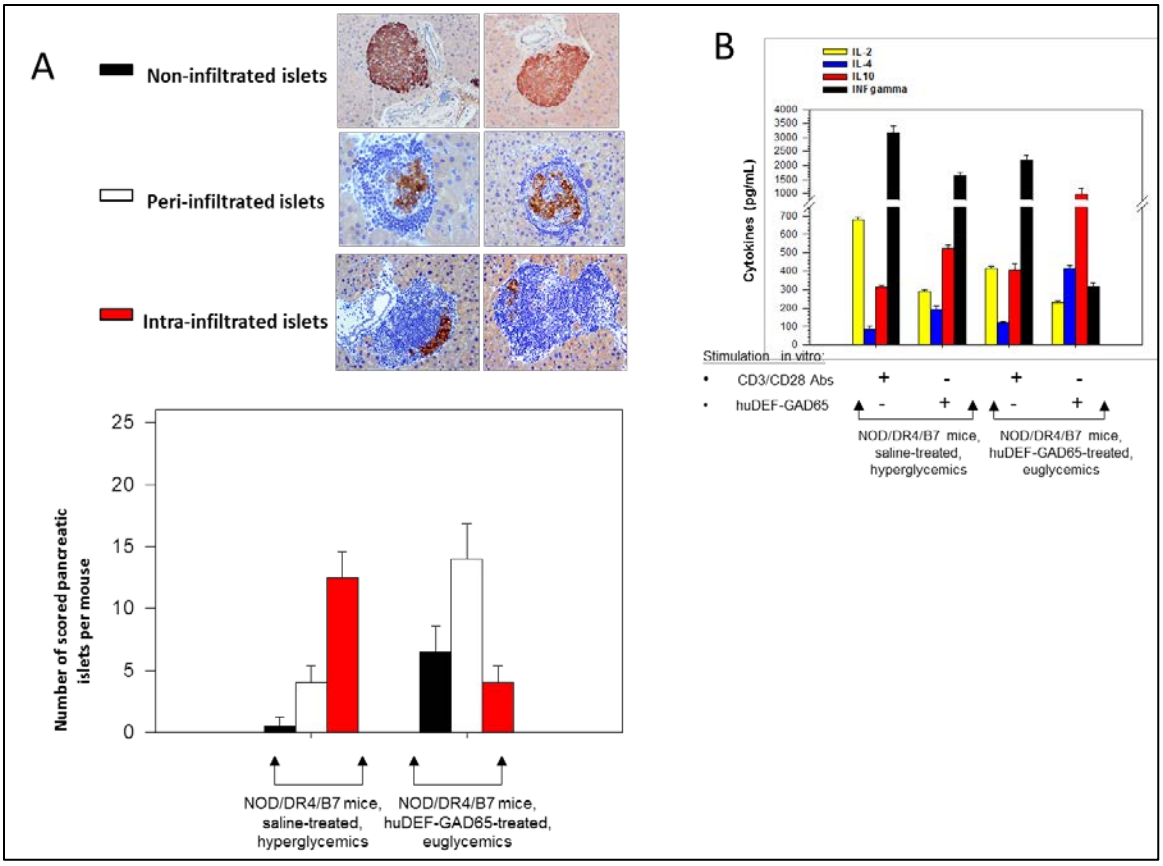

Figure 3.3 Pancreatic and T-cell analyses of NOD/DR4/B7 dTg mice treated or not with hu DEF-GAD65 reagent.

Panel A Comparative morphologic analysis of pancreatic islet infiltration in NOD/DR4/B7 dTg mice treated or not with hu DEF-GAD65 reagent as in figure 3.2, panel A. The pancreata from both groups of mice were analyzed at the end of experiment when mice reached 11 months of age. Some 20-25 β-islets per pancreas were analyzed from individual mice (n=5 mice/group). Of note, treated mice showed a significantly higher number of pancreatic peri-infiltrated islets than those in the control group. The small panel represents two representative, fully functional pancreatic β-islets lacking lymphocyte infiltration (upper panels), or peri-infiltrated β-islets (middle panels), or intra-infiltrated β-islets from the group of NOD/DR4/B7 dTg mice treated with hu DEF-

GAD65 reagent as in figure 3.2, panel A. Pancreata from both groups of mice were analyzed after treatment interruption, when mice reached 11 months of age. Shown in each panel are the HE staining of infiltrating lymphocytes (dark blue) and the presence of intra-islet insulin granules stained with a rabbit anti-Insulin-HRP conjugate. Of note, the peri-infiltrated islets show higher amount of insulin granules as compared with the intra-infiltrated islets (small panel showing representative pancreas used in the scoring process.) ***Panel B***, cytokine analysis in stimulated cells from pancreatic lymph nodes cultures from NOD/DR4/B7 dTg mice treated or not with hu DEF-GAD65 reagent. Of note, the CD4 T-cells from NOD/DR4/B7 dTg mice treated *in vivo* with hu DEF-GAD65 reagent secreted a significantly higher amount of IL-4 and IL-10, and lower amount of IFN-γ than those from saline-treated animals (control group). Shown are the mean values of cytokines ± SD for 4 individual mice in each group.

Chapter 4: Dissertation Summary and Discussion

T1D in no uncertain terms is a complex disease along with other immune disorders. This should not come as a surprise, understanding how complex the immune system is itself. The immune system helps us fight infections. At the same time, while adaptive or innate immune mechanisms take place to overcome a viral, bacterial or parasitic infection as well as to kill a cancerous cell, it needs to make sure it does not kill healthy functional cells and organs. Even though we have come a long way in our understanding of how the immune system works, it remains to be clear how these complex and very plastic immune cells are able to interact with each other to generate the immune makeup that is unique to each individual. Fully comprehending the principles that govern the immune system is our only way we could come with better methods to treat disease when said system goes haywire.

MHC class II has been shown extensively to be associated with T1D. Though, it is more relevant to consider the numerous genes that together contribute to disease susceptibility or resistance. Among the different MHC class II subclasses HLA-DR4 and DR3 have been shown, many times, to be strongly associated with T1D in various types of studies in association with DQ8 (33; 84; 86). The study performed by Wen et al. (115) suggests a regulatory potential of the class II MHC HLA-DR4. They generated a transgenic diabetes model on a C57BL/6 mouse expressing the co-stimulatory B7-1 in the pancreas and the human HLA-DR4 alone or associated with HLA-DQ8 in the absence of endogenous murine MHC class II ($Abb^{-/-}$) (115). They showed that the high incidence of diabetes in mice bearing only DQ8 gene (73%) was markedly reduced by the introduction of the DR4 gene (23%). However, around 25% of DR4/$Abb^{-/-}$/B7-1 mice yet became

diabetic whereas C57BL/6 DR4/B7-1(with endogenous MHC class II) mice did not become diabetic at all (115). They showed that the protective effects of HLA-DR4 are due to a switch in the cytokine response after activation with known autoreactive proteins in T1D such as GAD and insulin that is favorable to a Th2 type response when otherwise is a Th1 type response in a diabetic mouse.

By generating a NOD mouse carrying the human HLA-DR4 our goal was to generate a "humanized" mouse to study the role of DR4 in T1D. Remarkably, NOD/DR4 Tg mice were able to become diabetic only in the presence of the B7-1 costimulatory molecule expressed in the pancreas (described in chapter 3). The NOD/DR4/B7-1 mice became diabetic at a much higher frequency than the DR4/Abb$^{-/-}$/B7-1 (75% *vs.* 25%). This could be attributed to several *idd* genes from the NOD background and more importantly the MHC class II I-A^{g7} which are not expressed in the C57BL/6. In the study described in chapter 2 we aimed at determining the mechanisms of HLA-DR4 protection of diabetes in NOD mice.

Similar to Wen's et al. findings, we observed a reduced INFγ response upon stimulation of total splenocytes with known diabetogenic GAD65 and anti-CD3/CD28 indicating a reduced Th1 type of response. However, we did not detect a difference in IL-4. Nevertheless, downregulation of INFγ response suggests that DR4 protection is a result of more than one type of regulatory events such as an increase of Th2 and Tr1 cells which are potent suppressors of INFγ production by expression of regulatory cytokines such as IL-10 (121).

Tr1 cells are CD4+ T cells secreting high levels of IL-10. They have been reported to protect against T1D in NOD mice by downregulating activity of antigen

specific effector cells (10; 87). We found an increase in IL-10 release upon polyclonal stimulation. Interestingly, we also found a high frequency of $CD4^+$ T cell IL-10+ (Tr1) cells and CD4+Fox3+ Treg cells in 2 weeks old but not in adult 8 weeks old NOD/DR4 Tg mice compared to NOD non Tg littermates prone to T1D. These results suggest that DR4 protection is carried out during the developmental stages of the mouse immune system. During the first weeks of life the NOD develops autoreactive CD4+ and CD8+ T cells that escaped clonal deletion during thymic selection and are expanded in the periphery by pancreatic autoantigens presented by MHC (5). A higher number of T regulatory cells during the first weeks of the NOD/DR4 Tg mice life might out-weight the number of autoreactive T cells and arrest their activity early during the mouse development in the periphery in NOD/DR4 Tg mice, thus preventing the onset of diabetes.

The role of Foxp3 has been identified best in CD4+ T regulatory cells. Although much less is known about CD8+ T cells, recent reports have emerged describing CD8+Foxp3+ T cells with regulatory functions much as their CD4+ counterparts (11; 101; 102). Interestingly, immunosuppressive CD8+ T cells from heart transplant recipients have been described to express high levels of HLA-DR (24; 101), thus their activity is T cell specific much like a professional antigen presenting cells. They have been shown to promote T cell anergy in effector CD4+ T cells (24; 101; 102). They were shown to suppress CD4+ T cell activation by interfering with early steps in the TCR activation cascade upstream of ZAP70 phosphorylation (101). We found CD8+ T cells up-regulating the Foxp3 suppressogenic gene upon polyclonal stimulation in NOD/DR4 Tg mice but not in NOD non Tg littermates. Upregulation of Foxp3 expression in the

$CD8^+$ T cells of NOD/DR4 Tg mice but not the NOD non Tg littermates may explain the poor IFN-γ response with these mice, since Foxp3 can lower the IFN-γ synthesis by suppression of T-bet transcription required for IFN-γ synthesis (49; 71; 103)..

The nature of MHC class II-peptide presentation during the thymic selection of T-cells can shape the T-cell repertoire (43; 91). CD4+ T-cells in NOD/DR4 Tg mice are selected in thymus by antigen presentation of human HLA-DR*0401 MHC class II molecules in addition to the murine endogenous $I\text{-}A^{g7}$ molecules. The NOD non Tg mice are characterized by the lack of expression of I-E because of a deletion mutation in the I-Eα promoter region (5; 62). The HLA-DR4 molecule in NOD/DR4 Tg mice is expressed as a chimeric transgene HLA-DRA-IEα with the HLA-DRB1*0401-IEβ (44). The genes coded consist of the antigen binding domains of the HLA-DRA/DRB1*0401and the remaining portion of the murine $IE^d\alpha/IE^d\beta$ chains (44). Addition of a new MHC molecule like HLA-DR4 into the NOD mice could lead to the hypothesis that increased levels of MHC class II expression in the cell membrane results in skewing of the T cell pool in favor of CD4+ T cells. Observations in different hybrid mice bearing HLA-DR4 and $I\text{-}A^{g7}$ in association or not with $I\text{-}A^b$, described in figure 2.4 supports that hypothesis. Only the mice expressing HLA-DR4 in addition with murine MHC class II showed an increased frequency of CD4+ T cells. However, MHC class II expression is tightly regulated by different mechanisms. Levels of expression of MHC class II in the cell surface is carefully regulated by a ubiquitin ligase protein MARCH-1. In a report by McGehee et al. (68), they showed that mice expressing a mutant on the cytoplasmic region of MHC class II (K225R) that cannot be ubiquitinated have elevated levels of MHC class II on cell surface of dendritic cells. These mutant mice have no alterations in

the MHC-II presentation and the CD4+ T cell population remained unaffected compared to the wild type mice. Thus, even though there could be differential levels of MHC class II in NOD/DR4 Tg mice; other mechanisms are involved in dictating the CD4/CD8 T cell ratio. Such mechanisms must include the affinity of pMHC-TCR complex and the events that take place downstream of TCR signaling.

The $CD8^+$ T cell population was significantly reduced in the NOD/DR4 Tg mice and showed a slight increase in the population of $CD4^+$ T cell population in the thymus as well as in the periphery. The different CD4/CD8 peripheral T cell ratio in the NOD/DR4 Tg mice was mostly the result of a combined, altered CD4/CD8 thymic output and defective homeostatic mechanism. One suggested mechanism for these observations is that polymorphism occurring in higher rates in diverse regions of the MHC class II alleles according to a GWAS analysis determined the CD4/CD8 T cell ratio in favor of higher frequency of CD4+ T cells and it correlated to protection against diabetes (33). The results suggested that protection could be a result of selection of more regulatory rather than effector T cells (33). Our results confirmed this hypothesis. We observed an increase in regulatory (Tr1) and Foxp3+ $CD4^+$ T cells possibly enough to abrogate the incidence of diabetes in the NOD/DR4 Tg mice.

The master regulator for class II MHC is the class II transactivator (CIITA) (22; 72). CIITA is recruited to the X1, X2 and Y box elements in the MHC locus by a complex of transcriptional factors directly bound to the box elements (72). Levels of CIITA directly impact levels of MHC class II expression and are tightly regulated by post-translational modifications and factors that are mainly active in immune cells (72). After translation MHC class II undergoes posttranslational modifications that involve

loading with peptide in the MHC class II compartment (MIIC) (22; 72). Then, it is transported to the cell membrane. In the cell membrane the MHC class II is waiting to get in contact with CD4+ T cell receptors or it is ubiquitinated by the ubiquitin ligase membrane associated RING-CH I (MARCH-I). MARCH-I is active predominantly in immature dendritic cells but later blocked by CD83 in mature dendritic cells (72). Ubiquitination is an important mechanism cells employ to regulate trafficking of MHC class II, thus regulating levels of MHC class II expression via ubiquitination (68).

Some autoimmune disorders such as EAE are correlated to higher expression, lower or no expression of certain TCR Vβ families (51). Albeit, a recent attempt to determine a significant difference in the TCR Vβ repertoire of patients with T1D was made (111); the study was limited by the population size (15 children recently diagnosed with T1D). Nonetheless, they observed a significant increase of TCR Vβ4 in children recently diagnosed with T1D. Herein, we observed differences in the TCR Vβ repertoire that are similar to previous reports in studies done with several HLA transgenic mice including the C57BL/6 which does not express murine MHC class II but expresses the chimeric HLA-DRA1-I-Eα2 and DRB1*0401-I-Eβ2 (44) transgene (the mice used to generate the NOD/DR4). These results indicate that HLA-DR4 is capable of inducing changes in the TCRvβ repertoire. However, it remains to be answered what the clinical relevance of this finding is in the pathology of T1D. So far at this time no T1D Vβ family correlation has been reported yet in the NOD “wild type” mouse.

Clearly disease protection by HLA-DR4 is more complex and the result of several mechanisms that originate during thymic development of T cells. Such mechanisms include an increase in selection of regulatory CD4+ T cells including Tr1 and Foxp3+ T

cells early in the mouse life. Subsequently, driving the cytokine profile in the NOD/DR4 Tg towards a regulatory profile with lower levels of INFγ responses induced by an increased in regulatory IL-10 production.

There is no available mouse model similar to ours with a different human MHC class II such as DQ8 in a NOD background to confirm if indeed the results we see are unique to HLA-DR4. A NOD expressing human HLA-DQ8 and CD4 was generated by R. Flavell. Unfortunately these mice were not formally characterized.

It is important to consider the extreme similarities between the murine I-A^{g7} with the human HLA-DQ8 and the I-E with HLA-DR4. For this very reason the NOD is considered the best model for human T1D (5; 112). However, it also very important to note that NOD develops spontaneous diabetes whereas in humans the increased incidence over time has led researchers to look for environmental triggers (112). Our NOD/DR4 does not develop spontaneous diabetes but it does only after expression of the co-stimulatory molecule B7-1 in the pancreas. In humans pancreatic β cells are conditioned by the genetic makeup and environmental events to express inflammatory molecules such as TNFα and also MHC class I and co-stimulatory molecules B7-1/2 (112). We have not tested the possibility of inducing diabetes in our NOD/DR4 Tg mice by infection such as with coxsackievirus or other suspected triggers to induce T1D. Nonetheless, the NOD/DR4 mouse is an excellent tool to investigate the pMHC processing and presentation in the context of two different and seemingly counteractive MHC alleles.

The work described on chapter 3 was aimed at testing the therapeutic efficacy of a genetically-engineered pMHC class II-GAD65$_{271\text{-}285}$ chimera (DEF-GAD65 reagent) of human use to prevent the T1D onset. Thus, 75% of pre-diabetic humanized

NOD/DR4/B7 dTg mice treated with 8 small doses of hu DEF-GAD65 reagent remained euglycemic and showed a stabilized pancreatic lymphocyte infiltration for up to 6 months after treatment interruption.

Early during the course of T1D onset the NOD mice pancreatic cells are surrounded by lymphocytes such as dendritic cells and macrophages which then are followed by B and T lymphocytes (112). This stage has been denominated as peri-insulitis. At this point β cells are functional and there are no signs of destruction. By 4 to 6 months T cells progress to invade the islets and aggressively destroy it. This stage is known as insulitis. Remarkably, mouse β-cells proliferate due to expression of immflamatory molecules, however not enough to overcome T cell destruction.

Stabilization of T1D progression in NOD/DR4/B7 dTg mice occurred by mechanisms of Th2/TR-1 polarization. The ability of hu DEF-GAD65$_{271-280}$ reagent targeting only the GAD65$_{271-280}$-specific CD4 T-cells to inhibit the response of a polyclonal T-cell population as demonstrated in the CD3 stimulation experiments, is similar to that of its murine DEF-GAD65$_{217-230}$ reagent (60). Our results demonstrate that our treatment protected pancreatic β cells by preventing T cell destruction but did not eliminate thymic development of autoreactive T cells, as seen with the persistence of peri-insulitis. This suggests the need for a prolonged period of treatment.

Non-responder mice recovered glycemia levels very soon after treatment. This could be explained by the up-regulation of IL-10 in pancreatic islets which in turn reduces the levels of inflammatory molecules such as INFγ. INFγ has been shown to inhibit β-cells production of insulin (108). Hence, reducing the levels of INFγ in

pancreatic islets immediately could increase production of insulin enough to stabilize glycemia to basal levels.

We denominated this novel therapeutic concept as to “single-epitope bystander suppression”, due to the ability of a single peptide-specific reagent (like DEF) to suppress a polyclonal population of autoreactive cells through the stimulation/expansion of IL-10-secreting TR-1 suppressor cells at the site of inflammation (i.e., pancreas) (60). In conclusion, the results of this work strongly suggest that human DEF-like chimeras targeting diabetogenic T cells in the absence of systemic immune suppression may represent an alternative antigen-specific approach that can overcome the limitations of conventional antigen-specific reagents in T1D therapy.

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